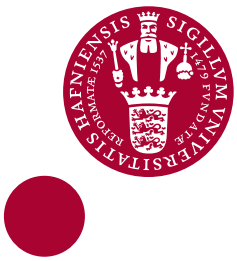

Molecular detection of foodborne pathogens

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Molecular Detection of Foodborne Pathogens

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Abstract

Salmonella and *Campylobacter* are recognised as some of the most important foodborne pathogens worldwide. Human infections have wide health and socioeconomic consequences. Lots of effort has been devoted to increase the knowledge on the prevalence, transmission routes and persistence of these pathogens in the food chain, in order to improve intervention strategies and make more effective the control of production lines and single food items. To serve this purpose, rapid and reliable detection and quantification methods are imperative.

The culture-based standard methods currently applied for detection and enumeration of *Salmonella* and *Campylobacter* are time-consuming and laborious. They lack specificity and do not enable detection of viable but non-culturable (VBNC) bacteria.

The focus of the present thesis has been development and validation of PCR-based detection methods for *Salmonella* and *Campylobacter*. A conventional PCR-based method for detection of *Campylobacter* in chicken carcass rinse following 20 h of enrichment in Bolton broth was successfully compared to the ISO standard culture-based method (10272) on 68 naturally infected chickens. The method was subsequently validated on artificially inoculated pig carcass swab and chicken carcass rinse samples in an international collaborative trial including 12 participating laboratories.

A real-time PCR-based method was developed from the conventional method, and this compared favourably to the ISO standard culture-based method (10272) on 12 artificially inoculated and 66 naturally infected chicken carcass rinse samples. The robustness, detection probability, precision, amplification efficiency and linear range of the method were evaluated on two different thermal cyclers, and the method was proven suitable for quantification. Furthermore, a linear relationship (R^2 = 0.94) was established between the threshold cycle values obtained in real-time PCR after enrichment and the culture-based enumeration. This real-time PCR method was validated according to the recommendations of the Nordic Organisation for Validation of Alternative Microbiological Methods (NordVal) in comparative and collaborative trials, and was approved for detection of *Campylobacter* in chicken neck skin, cloacal swab and boot swab samples.

A comparison study on probe chemistries for real-time PCR was performed on locked nucleic acid (LNA), minor groove binder (MGB), Scorpion and TaqMan probes. The LNA probe was shown to be the most sensitive probe chemistry in the real-time PCR assay for detection of

Campylobacter, producing the highest amplification efficiency. Choice of probe chemistry was found to impact the sensitivity of PCR assays, and should be considered in an optimisation strategy.

Finally, the real-time PCR method was evaluated for direct quantification of *Campylobacter* in chicken carcass rinse samples. A propidium monoazide (PMA) sample treatment was incorporated into the method, enabling PCR detection of viable *Campylobacter* only. A strong correlation (R^2 = 0.84) was obtained between the *Campylobacter* counts obtained by PMA-PCR and culture, indicating that the method presents as a reliable tool for producing accurate quantitative data on viable *Campylobacter*. DNA from dead cells was not detected by the proposed method, however, it recognised the infectious potential of the VBNC state, and would thus be able to assess the outcome and impact of increasingly applied post-slaughter reduction strategies.

A real-time PCR-based method for detection of *Salmonella* was optimised following a diversified approach to enable the shortest time of analysis possible. Positive effects of the optimisation strategy were observed from increasing 1) the sampling volume from the pre-enrichment, 2) the paramagnetic particles applied in the DNA extraction procedure, and 3) the amount of DNA template in the PCR. This method was subsequently validated according to the recommendations of NordVal in comparative and collaborative trials and was approved as an alternative method for detection of *Salmonella* in chicken neck skin, minced meat and pig carcass swabs.

In conclusion, this thesis presents the development and validation of real-time PCR methods for detection of *Salmonella* and *Campylobacter*, and for *Campylobacter* also quantification of viable cells only. These validated methods can help the dissemination and implementation of PCR-based methods in control laboratories, and thereby contribute to improved food safety.

Sammendrag (Danish)

Salmonella og *Campylobacter* er blandt de vigtigste fødevarebårne patogener. Humane infektioner har vidtrækkende sundhedsmæssige og økonomiske konsekvenser. Indsatsen for at tilvejebringe viden om prævalens, smitteveje og persistens i fødevarekæden er derfor intensiv, med det formål at forbedre interventionsstrategier og effektivisere kontrollen med fødevareproduktionen og de enkelte fødevarer. Til dette formål er det nødvendigt at have hurtige og sikre metoder til påvisning og kvantificering til rådighed.

I dag anvendes dyrkningsbaserede standardmetoder til påvisning og kvantificering af *Salmonella* og *Campylobacter*, som både er tids- og arbejdskrævende. De har mangler hvad angår specificitet, og er ikke i stand til at detektere levende-men-ikke-dyrkbare bakterier.

Denne Ph.d.-afhandlings fokus har været udvikling og validering af PCR-baserede detektionsmetoder til *Salmonella* og *Campylobacter*. En konventionel PCR-metode til at detektere *Campylobacter* i kyllingerens efter 20 timers opformering i Bolton bouillon blev med tilfredsstillende resultat sammenlignet med den dyrkningsbaserede ISO-standard (10272) på 68 naturligt inficerede kyllinger. Metoden blev efterfølgende valideret på spikede svinesvaber prøver og kyllingerens i en international kollaborativ test med 12 deltagende laboratorier.

En real-time PCR-baseret metode blev udviklet fra den konventionelle metode, og denne blev sammenlignet succesfuldt med den dyrkningsbaserede ISO-standard (10272) på 12 spikede og 66 naturligt inficerede kyllingerens-prøver. Metodens robusthed, detektionssandsynlighed, præcision, amplifikationseffektivitet og lineære rækkevidde blev evalueret på to forskellige thermocyklere, og den blev fundet egnet til kvantificering. Ydermere kunne der etableres en lineær sammenhæng (R^2 = 0,94) mellem de opnåede Ct-værdier og den dyrkningsbaserede kvantificering.

Denne real-time PCR metode blev valideret i henhold til anbefalingerne fra den Nordiske Organisation for Validering af Alternative Mikrobiologiske Metoder (NordVal) i komparative og kollaborative studier, og blev godkendt til detektion af *Campylobacter* i kyllingehalsskind, kloaksvabere og sokkeprøver.

Et real-time PCR sammenligningsstudie blev udført med probekemierne locked nucleic acid (LNA), minor groove binder (MGB), Scorpion og TaqMan. LNA proben viste sig at være den mest sensitive til detektion af *Campylobacter*-DNA, og samtidig var det også her den højeste

amplifikationseffektivitet blev opnået. Valg af probekemi blev påvist at have indflydelse på PCR-sensitiviteten og burde overvejes i optimeringssammenhæng.

Endelig blev real-time PCR-metoden evalueret mhp. direkte kvantificering af *Campylobacter* i kyllingerens. Med det formål kun at detektere levende *Campylobacter* celler blev en propidiummonoazid prøvebehandling inkorporeret i metoden. En stærk korrelation mellem kvantificering af *Campylobacter* vha. PCR og dyrkning blev fundet (R^2 = 0,84), hvilket understregede metodens anvendelighed til at tilvejebringe nøjagtige kvantitative data. DNA fra døde celler blev ikke detekteret, men metoden var i stand til at anerkende det infektiøse potentiale af levende-men-ikke-dyrkbare celler, og vil derfor kunne anvendes til at evaluere resultatet og dermed effekten af diverse reduktionsstrategier.

En real-time PCR-baseret metode til detektion af *Salmonella* blev optimeret efter et flerstrenget princip, for at reducere analysetiden mest muligt. Positive effekter sås som følge af et forøget 1) prøvevolumen fra præopformering, 2) indhold af paramagnetiske partikler i DNA-ekstraktionen og endelig 3) DNA-volumen i PCR. Metoden blev efterfølgende valideret i komparative og kollaborative studier i henhold til anbefalingerne fra NordVal, og blev godkendt som en alternativ metode til detektion af *Salmonella* i kyllingehalsskind, hakket kød og svinesvabere.

Afhandlingen præsenterer udviklingen og valideringen af real-time PCR-baserede metoder til detektion af *Salmonella* og *Campylobacter*, og for *Campylobacter* ydermere til kvantificering af levende bakterier. De validerede metoder kan lette udbredelsen og implementeringen af PCR-baseret detektion i kontrollaboratorier, og dermed bidrage til forbedret fødevaresikkerhed.

List of manuscripts

The present thesis is build upon the following manuscripts with permission from authors and copyright holders.

I: Josefsen, M.H., P.S. Lübeck, F. Hansen, and J. Hoorfar. 2004. Towards an international standard for PCR-based detection of foodborne thermotolerant campylobacters: interaction of enrichment media and pre-PCR treatment on carcass rinse samples. J. Microbiol. Methods 58:39-48.

II: Josefsen, M.H., N. Cook, M. D'Agostino, F. Hansen, M. Wagner, K. Demnerova, A.E. Heuvelink, P.T. Tassios, H. Lindmark, V. Kmet, M. Barbanera, P. Fach, S. Loncarevic, and J. Hoorfar. 2004. Validation of a PCR-based method for detection of foodborne thermotolerant campylobacters in a multicenter collaborative trial. Appl. Environ. Micrbiol. 70:4379-4383.

III: Josefsen, M.H., N.R. Jacobsen, and J. Hoorfar. 2004. Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of foodborne thermotolerant campylobacters. Appl. Environ. Micrbiol. 70:3588-3592.

IV: Krause, M., M.H. Josefsen, M. Lund, N.R. Jacobsen, L. Brorsen, M. Moos, A. Stockmarr, and J. Hoorfar. 2006. Comparative, collaborative, and on-site validation of a TaqMan PCR method as a tool for certified production of fresh, *Campylobacter*-free chickens. Appl. Environ. Microbiol. 72:5463-5468.

V: Josefsen, M.H., M. Krause, F. Hansen, and J. Hoorfar. 2007. Optimization of a 12-hour TaqMan PCR-based method for detection of *Salmonella* bacteria in meat. Appl. Environ. Microbiol. 73:3040-3048.

VI: Löfström, C., M. Krause, M.H. Josefsen, F. Hansen and J. Hoorfar. 2009. Validation of a same-day real-time PCR method for screening of meat and carcass swabs for *Salmonella*. BMC Microbiol. 9.

VII: Josefsen, M.H., C. Löfström, H.M. Sommer and J. Hoorfar. 2009. Diagnostic PCR: Comparative sensitivity of four probe chemistries. Mol. Cell. Probes 23:201-203.

VIII: Josefsen, M.H., C. Löfström, T.B. Hansen, L.S. Christensen, J.E. Olsen and J. Hoorfar. 2009. Quantification of viable *Campylobacter* in fresh chicken carcass rinse by real-time PCR and propidium monoazide as a novel strategy for risk assessment. *In Preparation* for Appl. Environ. Microbiol.

Book chapter

Josefsen, M.H., C. Löfström, K.E.P. Olsen, K. Mølbak and J. Hoorfar. 2009. *Salmonella*. In: Molecular detection of human pathogens. Don Liu (Ed.). Taylor & Francis CRC Press. Submitted.

Abbreviations

AC	accuracy
AE	amplification efficiency
AFNOR	Association Francaise de Normalisation
AHB	Abeyta-Hunt-Bark
AOACI	Association of Official Analytical Chemists International
ATCC	American Type Culture Collection
a_w	water activity
BA	blood agar
BAM	bacteriological analytical manual
BB	Bolton broth
BHI	brain heart infusion
BHQ	black hole quencher
Bp	base pairs
BPW	buffered peptone water
BSA	bovine serum albumin
CCUG	Culture Collection University of Göteborg
CD	committee draft
CDT	cytolethal distending toxin
CFU	colony forming units
CI	confidence interval
COR	concordance odds ratio
CPS	capsular polysaccharide
Ct	threshold cycle
CV	coefficient of variation
ΔRn	normalised reporter signal
DFFE	Directorate for Food, Fisheries and Agri Business
DFVF	Danish Food and Veterinary Research
DIN	Deutsches Institute für Normung
DIS	draft international standard
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
DTU	Technical University of Denmark
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetraacetic acid
EFSA	European Food Safety Agency
EMA	ethidium monoazide
EU	European Union
FAM	carboxy fluorescein
FBP	ferrous sulphate, sodium metabisulphite, sodium pyruvate
FDA	US Food and Drug Administration
FDIS	final draft international standard

FN	false negative
FP	false positive
G+C	guanine+cytosine
IAC	internal amplification control
IgA	immunoglobulin A
inv	gene cluster encoding invasins in *Salmonella*
IRMM	Institute for Reference Material and Measurements
ISO	International Standard Organisation
KF	KingFisher
LB	lysegenic broth
LNA	locked nucleic acid
LPS	lipo polysaccharide
LUX	light upon extention
mCCDA	modified cefoperazone charcoal deoxycholate agar
MGB	minor groove binder
MHB	Mueller Hinton broth
MKTTn	Muller-Kaufmann tetrathionate-novobiocin
NA	negative agreement
NCTC	National Collection of Type Cultures
ND	negative deviation
NFQ	non-fluorescent quencher
NMKL	Nordic Committee on Food Analysis
NordVal	Nordic Organisation for Validation of Alternative Microbiological Methods
NTC	nontemplate control
OR	odds ratio
PA	positive agreement
PAI	pathogenicity island
PB	Preston broth
PCR	polymerase chain reaction
PD	positive deviation
PhHV	phocine herpesvirus
PI	propidium iodide
PMA	propidium monoazide
PNC	processing negative control
Q	quencher
R	reporter
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RVS	Rappaport Vassiliades soy
s^2	sample variance
SD	standard deviation
SDS	sequence detection system
SE	sensitivity
SOP	standard operating procedure

SP	specificity
SPI	*Salmonella* pathogenicity island
SSI	Statens Seruminstitut
TAMRA	carboxy tetramethyl rhodamine
Tth	*Thermus thermophilus*
TP	true positive
TSB	tryptone soya broth
T3SS	type 3 secretion system
U	unit
UNG	uracil-*N*-glycosylase
VBNC	viable but non-culturable
WHO	World Health Organisation

Contents

1. Introduction

Foodborne infections are a serious problem, affecting public health worldwide. *Salmonella* and *Campylobacter* are two of the most important foodborne pathogens (17, 21, 170). The discomfort, possible sequelae, clinical cost and working hours lost following infection, presents a serious problem from both a health and a socioeconomic perspective. *Salmonella* has been estimated to be responsible for 1.3 billion human infections annually (136), and in the majority of the developed word, *Campylobacter* infections have exceeded the number of *Salmonella* infections and become the leading cause of bacterial gastrointestinal disease (134).

Both pathogens are zoonotic agents, transmitted from animals and animal products to humans. The presence of these pathogens in the food chain entails the necessity of intervention strategies, as well as reliable and efficient control of production lines and single food items. Methods for detection, enumeration and characterisation are imperative to improve knowledge on prevalence, route of transmission and persistence in the food chain, as well as to enhance the control and intervention strategies of *Salmonella* and *Campylobacter*, and to ensure the safety of the food items available for consumers.

Until recently, microbiological methods for detection and enumeration of *Salmonella* and *Campylobacter* relied solely on culture. Standardised reference culture methods for these organisms have been issued by the national and international standardisation organisations, and are applied in control laboratories throughout the world. The culture-based approach is however not optimal, due to poor specificity, lack of detection of viable but non-culturable (VBNC) cells, and a time-consuming and labour-intensive nature. From a food safety point of view, these culture-based methods fail the purpose of producing rapid and reliable results, which can ensure the microbiological safety of products being released to the consumer.

PCR has revolutionised microbiological methods for detection of foodborne pathogens. Compared to culture-based methods, PCR offers valuable advantages regarding speed, sensitivity, specificity, cost and potential for automation (173). However, a lack of standardisation, validation and ability to differentiate between viable and dead microorganisms, has hampered the implementation of PCR-based methods in the control laboratories (78).

Focusing on *Salmonella* and *Campylobacter*, the objective of this thesis was to improve molecular-based diagnostic tools for risk assessment. To enable their application in assessing the effect of reduction strategies, and use in control of the food chain, and thereby ensure a rapid and reliable detection of these pathogens.

This thesis describes the development of molecular-based methods for detection of *Salmonella* and *Campylobacter*. It is build upon eight manuscripts regarding the evolving process of identifying, developing, optimising and finally validating PCR-based methods for *Salmonella* and *Campylobacter*. The methods proposed in this thesis have been developed in close collaboration with the Danish meat industry, with their requirements kept in mind to ensure the justification of the methods in the relevant control laboratories.

2. Background on *Salmonella* and *Campylobacter*

2.1 *Salmonella*

2.1.1 Classification, biology and clinical features

The taxonomic classification and nomenclature of the *Salmonella* genus has been subject to a number of rearrangements over the years, as a natural consequence of improved methods for determining the true relatedness of this group of bacteria. Currently the *Salmonella* genus consists of the two species *S. enterica* and *S. bongori*, two lineages suspected to have diverged early in evolution. *S. enterica* is further divided into the following six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*.

Subspecies are seperated into serovars according to the antigenic White-Kauffmann-Le Minor scheme (72), based on somatic (O), flagellar (H) and capsular (Vi) antigens. Today a total of 2579 serovars are recognised, the vast majority (>99.5%) belonging to the *S. enterica* species (72). Of these, more than half belong to *S. enterica* subsp. *enterica*, which are mainly associated with warm-blooded animals, while the remaining subspecies predominantly are found in cold-blooded animals and the environment (171).

Salmonella are Gram-negative facultative anaerobic rods belonging to the *Enterobactericeae* family. The size of the rods ranges from 0.7-1.5 μm - 2.2-5.0 μm, producing colonies of approximately 2-4 mm in diameter. They are non-spore forming, oxidase negative and catalase positive and most members of the genus are motile by petrichous flagella.

Salmonella are known to be quite resilient towards environmental influences, and prolonged persistence in hostile surroundings has been reported (49, 86, 169). This enhanced survival promotes the transmission of *Salmonella* from the environment to new hosts (187). The optimal growth temperature for *Salmonella* is 37°C, but the temperature range is quite broad (7-45°C), and preconditioning of cells can result in growth at extreme temperatures at both ends of the scale. Growth of *Salmonella* in various foods has been observed at 2-4°C (50, 62, 167).

Human *Salmonella* infection manifests it self as gastroenteritis, enteric (typhoid) fever or in rare cases systemic infections. Typhoid fever is caused by the host restricted serovars *S. enterica* subsp. *enterica* serovar Typhi and serovar Paratyphi A, B and C, with humans being the main reservoir, and the general route of transmission being faecal-oral.

Exposure to non-typhoid *Salmonella* from contaminated foods or from environmental sources can result in infections, depending on a number of factors such as serovar, number of bacteria ingested, vehicle of infection, and microbial environment of the gut. A low infectious dose has been reported for *Salmonella*, particularly in association with deficiencies in host defences, or if ingested in a vehicle protecting the bacteria from the detrimental effects of the gastric acid (119).

Salmonella infection often presents as an acute gastrointestinal illness. The onset of diarrhoea usually occurs within 6 to 48 hours after ingestion of the contaminated food, but ingestion of a high dose of bacteria can shorten the incubation period to only a few hours. Besides diarrhoea (that may be blood-containing) other common symptoms include abdominal pain or cramps, fever, chills, nausea, vomiting, pain in the joints, headache, myalgia, general malaise and weight loss. In uncomplicated cases, fever usually resolves within 48 to 72 hours, and the average time to recovery is one to two weeks.

2.1.2 Epidemiology

Salmonella continues to be an important zoonotic pathogen, affecting the health of both humans and animals on a global scale, thus having wide socioeconomic impact. *Salmonella* has been estimated to be responsible for 1.3 billion human infections annually. Data from the World Health Organisation (WHO) estimates that typhoid fever accounts for 21.7 million of these (47). Currently, in the US, 40,000 human *Salmonella* infections are reported annually, however, the true incidence is estimated to 1.4 million a year, resulting in 600 deaths (20), with an estimated cost of 3 billion $ (21). In the European Union 151,995 confirmed cases of human *Salmonella* infection were reported to the European Food Safety Agency (EFSA) in 2007 (8).

From 2000 to 2002 a survey of the global distribution of *Salmonella* serovars proved *S*. *enterica* subsp. *enterica* serovar Enteritidis to be the far most common serovar isolated from humans, accounting for 65% of all isolates. This tendency was even more pronounced in Europe where *S*. *enterica* subsp. *enterica* serovar Enteritidis was associated with 85% of all human cases (70). The second most frequently isolated serovar from human infections was *S*. *enterica* subsp. *enterica* serovar Typhimurium (12%) followed by serovar Newport (4%) (70). The dominance of these serovars could be attributable to their invasive behaviour in important food animals (83). Since the majority of human isolates have similar serovars, efficient source tracing in foodborne *Salmonella* outbreaks has to rely on further characterisation of isolates. Additional discriminatory typing methods like phage typing,

antimicrobial susceptibility testing and pulsed-field gel electrophoresis is commonly applied for this purpose.

2.1.3 Salmonella in the food chain

The principal habitat of *Salmonella* is the intestinal tract of warm-blooded animals. The natural excretion into the surrounding environment, where the bacteria are able to persist, explains the ubiquity of *Salmonella*. The intensive production of food animals in the agricultural industry, and the transmission of *Salmonella* into this production through contaminated feed and animal supply flocks, wild life reservoirs and the environment, sustains this existence (48).

Commonly *Salmonella* colonises the intestinal tract of food animals without causing symptoms of illness, and contamination of meat and meat products with intestinal contents during slaughter can and will occur. The establishment of *Salmonella*, combined with intensive therapeutic and growth promoting use of antimicrobial compounds, in the agricultural industry, has prompted problems with antibiotic resistance. The antibiotics applied in the primary production of food animals, selects for strains that confer resistance towards them, and during subsequent human infection with these strains, options of treatment will exclude these antibiotics. Treatment failure is currently a problem in both developed and developing countries (45).

The majority of human *Salmonella* infections are contracted through consumption of food of animal origin. Especially meat, poultry, eggs and milk are reported as vehicles of transmission (21). A wide variety of foods are however known to be associated with *Salmonella* infections, including fresh and dry fruits and vegetables, where contamination can occur during fertilisation, irrigation, harvesting or handling under non-optimal hygiene conditions (82). The transmission of *Salmonella* is cyclic between humans, animals, food items and environmental sources, and the basic routes are depicted in Figure 1.

Human *Salmonella* infections are mainly sporadic, however foodborne outbreaks do also occur regularly (21). Foods commonly associated with outbreaks of *Salmonella* are: Milk-based products (chocolate, cheese, salads, ice cream, etc.), sliced cold meat, vegetables and fruits (48). Pasteurised milk contaminated with *S. enterica* subsp. *enterica* serovar Typhimurium was responsible for one of the largest outbreaks with 16,284 confirmed cases in the US in 1985 (102), and recently peanut butter and peanut butter containing products contaminated with *S. enterica* subsp. *enterica* serovar Typhimurium have been responsible

for 741 human infection in US up to April 2009 (23). In Denmark, an ongoing outbreak with an unidentified source has caused more than 1300 human infections so far with *S. enterica* subsp. *enterica* serovar Typhimurium (24).

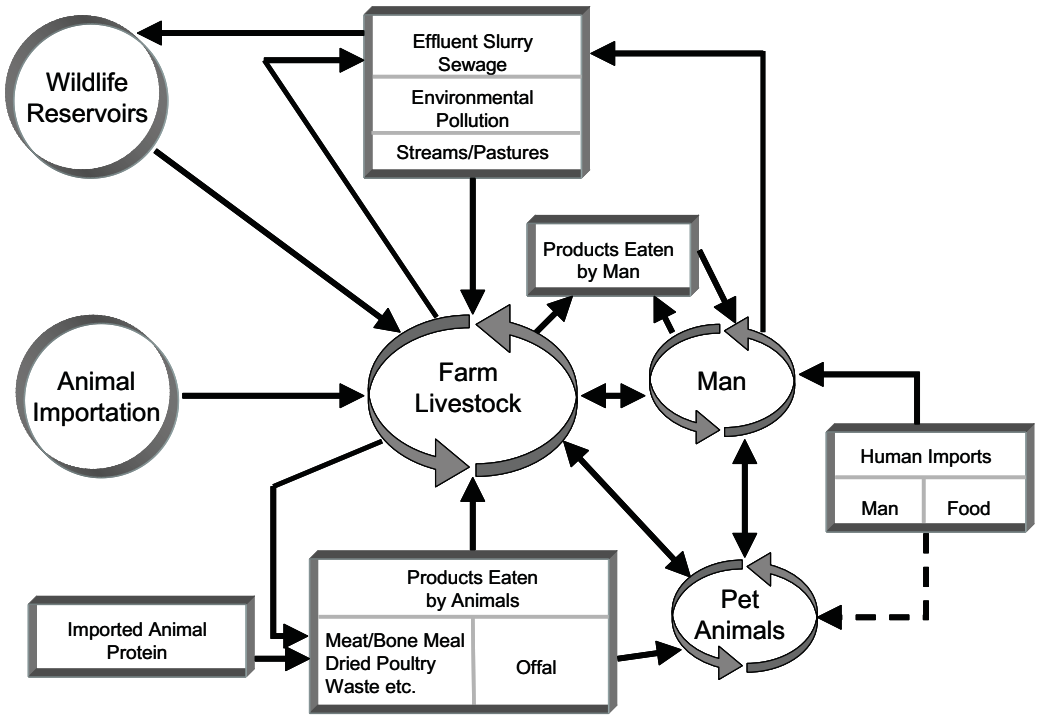


Figure 1. Basic transmission routes of *Salmonella*. Adapted with permission from Linton (1983) (107).

2.2 *Campylobacter*

2.2.1. Classification, biology and clinical features

The genus *Campylobacter* has undergone profound taxonomic rearrangements over the years. Today it comprises 18 species and 6 subspecies (54). *Campylobacter* belong to the family *Campylobactereraceae*, which also includes *Arcobacter*, *Bacteroides ureolyticus* and *Sulfospirillum* (164).

Campylobacter cells are mostly slender, spirally curved rods ranging from 0.2 - 0.8 μm in width and 0.5 - 5 μm in length. They are generally motile with a single polar flagellum at one or both ends giving rise to a very characteristic corkscrew-like motion. They are Gram negative and non-spore forming. Most members of the genus grow under microaerobic conditions, and do not ferment or oxidise carbohydrates, but obtain energy from amino acids or tricarboxylic acid cycle intermediates (158, 176). Optimal growth temperature for *Campylobacter* is 42°C, and growth does not occur below 30°C. Heating (decimal reduction time at 55°C is 1 min), drying and freezing reduces the number of viable cells substantially

(33, 137). Hence, no proliferation of these bacteria is observed in food items either during processing or storage. *Campylobacter* differs from other foodborne pathogens in being quite fastidious in their growth requirements, and extremely sensitive towards environmental stress. This pathogen seems to lack adaptive mechanisms, as well as the ability to recognise and respond to various environmental stress influences (138).

Some strains of *Campylobacter*, as many other microorganisms, are known to enter a viable but non-culturable state (VBNC) to survive for prolonged periods of time in unfavourable environments like water (146). The epidemiological significance of this cell state has been a subject of debate, as some studies have found the VBNC organisms unable to colonise newly hatched chicks (73, 117, 193, 194), while others demonstrate a regained culturability after in vivo passage in mice (29). In a study by Federighi et al. (1998) reversion of VBNC cells to culturable cells was experimentally demonstrated in a mouse model (61), and Klancnik et al., (2009) published a study showing diminished persistence and virulence properties, but retained infectious potential, of the VBNC organisms, in a mouse model (93). Since these findings do not lead to a conclusive determination of the infectious potential of the VBNC state, it is essential that diagnostic methods for foodborne *Campylobacter* allow for detection and quantification of this state as well (Manuscript IV).

A low infective dose has been reported for *C. jejuni*. Human trials demonstrated that as little as 500 organisms could cause infection. Development of illness was, however, first seen at approx. 10^4 organisms, depending on the immune status of the host, and did not show a clear dose relation (32). Epidemiological observations in humans indicated that the majority of infections were associated with ingestion of a low number of *Campylobacter* that colonise and multiply inside the host (184).

The symptom of a foodborne *Campylobacter* infection is usually profuse watery diarrhoea (sometimes blood-containing), accompanied by one or all of the following; nausea, abdominal pain and cramping, malaise, headache and fever (14, 84, 125). The pathogenesis reflects the virulence of the infecting strain, as well as the susceptibility of the host (138). The illness is usually self limiting with duration of 2-10 days, but severe cases are treated with erythromycin and ciprofloxacin (183). A rare but serious sequelum of *Campylobacter* infections is Guillain-Barré Syndrome, a neurological paralysis originating from a host immune response towards *Campylobacter*. The autoimmune response is an effect of bacterial antigenic compounds mimicking the neurological tissue antigens of the host (183).

2.2.2 Epidemiology

Campylobacter has become the leading cause of bacterial gastrointestinal disease in large parts of the developed world. In the European Union, 200,507 confirmed cases of human *Campylobacter* infections were reported to EFSA in 2007, and for most member states an increased incidence was observed compared to previous years (8). In 2007, 3868 cases of *Campylobacter* infections were registered in Denmark (9). The frequency of infection, duration of illness and possible sequelae makes *Campylobacter* a highly important pathogen from a socio-economic perspective. In the US alone, the economic impact due to clinical costs and lost working hours has been estimated to an annual sum of 1.3 – 6.2 billion $ (65).

Campylobacter jejuni subsp. *jejuni* and *C. coli* are by far the most important foodborne human pathogens in the genus, and accounts for more than 95% of all human clinical isolates world wide (100). However, this figure might be biased, since traditional detection methods favour isolation of these species compared to other potentially pathogenic, but more sensitive *Campylobacter* species (Manuscript I). The above-mentioned two thermotolerant species including *Campylobacter lari*, which is also a relevant foodborne species, will albeit be the focus of this thesis, and in the following text the term *Campylobacter* will refer to them unless otherwise stated.

2.2.3 Campylobacter in the food chain

Campylobacters are ubiquitous and are considered commensals of poultry and other avian species, as well as other production and domesticated warm-blooded animals. The basic transmission routes of *Campylobacter* are shown in Figure 2, and as illustrated the primary route of human infection is suspected to be foodborne. Thus, *Campylobacter* is considered to be a zoonotic agent transmitted from animals and animal products to humans.

Outbreaks of *Campylobacter* are rarely seen. Most of the reported cases are sporadic, including only a single family or community member at any one time. The rare foodborne outbreaks related to *Campylobacter* are predominantly caused by contaminated poultry, milk or drinking water (8, 9). In the last five years (2005 to 2009) the largest outbreaks caused by *Campylobacter*, in Denmark, were waterborne or related to cross-contamination from raw chicken to ready-to-eat food items (9). Even though sources of human infection are rarely identified, case control studies point towards various risk factors: consumption and handling of undercooked or raw poultry (cross-contamination), travelling, contact with

young domestic animals, unpasteurised milk, swimming in surface waters and consuming barbecued meals (5, 11, 14, 84, 127, 162, 183).

Especially poultry is suspected to be a major source of human infections. This assumption was strengthened during the Belgian dioxin crisis in 1999. All domestically produced poultry and poultry products were withdrawn from the marked due to dioxin contaminated feed, resulting in a 40% reduction in human *Campylobacter* infections in the same period of time (178).

In 2007, 27% of Danish broiler flocks were positive for *Campylobacter*. The seasonal variation, peaking in July and August, resulted in a prevalence of positive broiler flocks per month ranging from 9% in April to 56% in August. This seasonal pattern is reflected in human *Campylobacter* infections as shown in Figure 3. Regarding chilled Danish broiler meat at retail, 30% was positive for *Campylobacter* in 2007 (9). In 2007, EFSA reported an average of 26% of fresh poultry meat at retail to be positive for *Campylobacter*. *Campylobacter* was also commonly detected in live poultry, pigs and cattle (8).

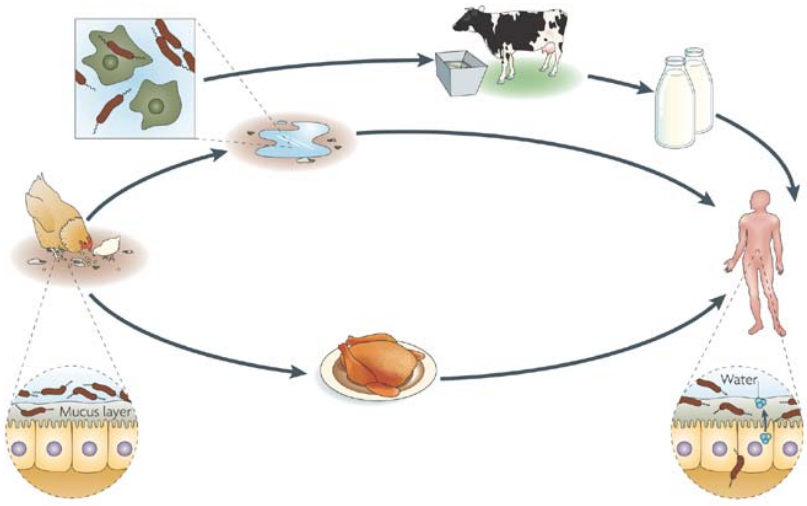


Figure 2. Basic transmission routes of *Campylobacter*. Reprinted with permission from Young et al., 2007 (192).

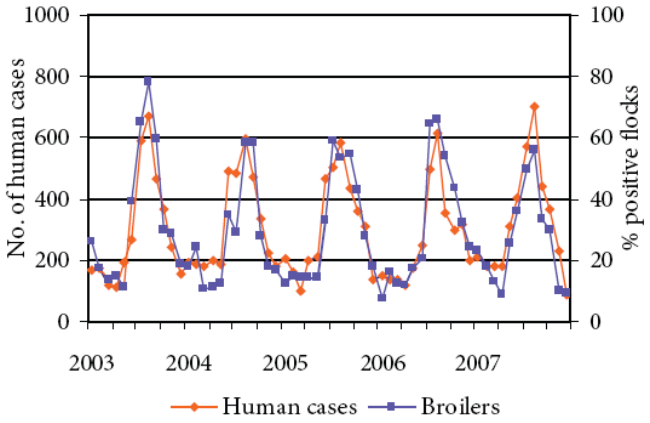


Figure 3. Monthly prevalence of *Campylobacter*-positive broiler flocks and number of human *Campylobacter* infections in Denmark from 2003 to 2008 (9).

3. Culture-based detection

3.1 *Salmonella*

The detection and isolation procedures for *Salmonella* are in principal similar in the various standard methods recommended by the US Food and Drug Administration's (FDA's) Bacteriological Analytical Manual (BAM), Association of Official Analytical Chemists International (AOACI), Nordic Committee on Food Analysis (NMKL) as well as the International Organisation for Standardisation (ISO), however, the media used in the different steps differ (7, 16, 18, 173). In the present thesis, the Nordic NMKL standard culture method (No. 71) was used as a reference method (Manuscript V and VI, Figure 4).

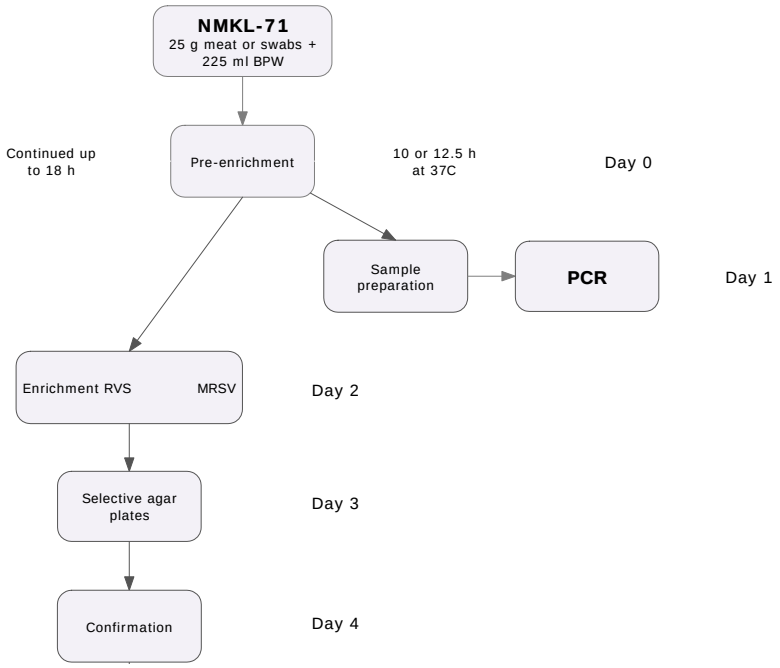


Figure 4. The NMKL-71 and PCR procedures for detection of *Salmonella* applied in Manuscript VI.

Food, animal feed and environmental samples usually contain low numbers of potentially stressed *Salmonella* bacteria and therefore an enrichment procedure is needed. The conventional routine analysis methods for isolating *Salmonella* rely on four steps, the first being pre-enrichment in non-selective media to aid the recovery of sublethally injured *Salmonella* (64). Even though *Salmonella* is viable and able to cause disease under the right conditions, the organisms can easily be killed if they are grown under selective pressure, such as high temperature or in the presence of chemical additives (180). It has however been suggested that pre-enrichment in non-selective enrichment media can mask the presence of *Salmonella* by allowing growth of competing microflora (168).

Buffered peptone water (BPW) is recommended for pre-enrichment in most standard methods, and it was also shown to be slightly superior to brain heart infusion broth and tryptone soya broth in supporting the resuscitation and growth of *Salmonella* after 6 and 8 hours of pre-enrichment in the present thesis (Manuscript V). Addition of growth-promoting or selective reagent directly to the BPW was also attempted, but this did not provide *Salmonella* with a competitive edge (Manuscript V). Based on the results obtained in this study it was speculated that a delayed addition of selective reagents to the BPW could be an alternative, in order to allow an initial recovery of stressed and injured *Salmonella* cells. Even though slightly improved real-time PCR results were obtained by addition of novobiocin (20 and 50 mg/L) and brilliant green (10 mg/L) to the BPW, the advantages of this was not sufficient to apply them, and thereby deviate from the enrichment procedure of the standard method, making retrospective culture-confirmation of PCR-results difficult (Manuscript V). Addition of malachite green has been reported to have both inhibitory and promotive effects on the growth of *Salmonella* in other studies (34, 88), and brilliant green has also been found to be inhibitory (34). Novobiocin addition has been reported to increase the recovery of *Salmonella* from faecal samples (87).

The second step is enrichment in selective media to increase the levels of *Salmonella*, thus enabling detection on selective agar plates. This is accomplished by inhibiting the growth of accompanying microflora by the addition of different selective agents in the media. The most commonly used media for selective enrichment are Rappaport-Vassiliades soy broth, selenite cysteine broth and tetrathionate broth. Modified semisolid Rappaport-Vassiliades is another selective media particularly useful for detecting salmonellae in feces and environmental samples (52). It is based on the ability of salmonellae to migrate through the selective medium ahead of competing motile organisms, thus producing opaque halos of growth.

Third step in the analysis method is isolation on selective agar plates by streaking out the selective enrichment broths on selective solid media to obtain isolated colonies. The growth of other bacteria is selectively repressed by the addition of inhibitory compounds and salmonellae are differentiated from other bacteria e.g. by the production of H_2S or acid from sugar. It is recommended that at least two different media be employed, since different serotypes can have varying sensitivity towards the different selective agents applied in the agar (157). Commonly employed media include brilliant green, xylose lysine Tergitol-4, bismuth sulphide, Hektoen enteric, xylose lysine deoxycholate and Rambach agars. Recently, several chromogenic agars have been compared favourably to these, being more sensitive and specific than the common agars (40, 57, 68, 111, 141, 174). Adding to the advantages of

the chromogenic agar is that the need for subsequent confirmation of isolates by subculture and biochemical test is reduced, since identification from the primary medium is possible.

Finally, confirmation of presumptive *Salmonella* isolates is performed by biochemical tests and serotyping. Presumptive colonies are inoculated on non-selective plates in order to obtain isolated colonies that can be characterised. Several biochemical tests are then employed, i.e. triple sugar iron agar, mannitol, urea, ornithin decarboxylase and lysine decarboxylase (18). A serological verification is performed by determining the antigenic composition. The antigens are classified as somatic (O) and flagellar (H) and are detected by an agglutination test using polyvalent antisera.

Human gastroenteritis caused by *Salmonella* is diagnosed by culturing fecal samples. Normally, isolation is accomplished by direct plating on selective plates from fecal samples containing high numbers of target bacteria (71). An enrichment step in selenite broth has been reported to increase the sensitivity of the culture method, but this has to be weight against the additional 24 h needed, until a diagnostic answer can be given.

3.2 *Campylobacter*

Detection and isolation procedures for *Campylobacter* differ slightly more than for *Salmonella* regarding the media, selective agents and incubation temperatures applied in the various standard methods from ISO, BAM and NMKL. However, the main principles of the standard methods are similar, and imply direct spreading onto selective agar plates of samples with a presumed high number of *Campylobacter* (fecal), and enrichment prior to selective isolation of samples a) presumed to harbour low numbers of *Campylobacter*, or b) samples containing a relatively large fraction of cells injured due to processing or unfavourable environmental conditions (12, 13, 85).

For food samples and other samples where a low number/injured *Campylobacter* cells are suspected to be present in a highly mixed background flora, an enrichment step is necessary prior to isolation on selective agar plates. For fecal samples drawn after the acute stage of the infection an enrichment step can likewise be necessary. Some of the most frequently employed enrichment broth media for *Campylobacter* are Bolton, Preston, Park-Sanders and Exeter. Since *Campylobacter* is sensitive towards peroxides, radical scavengers like horse/sheep blood and charcoal are often included in these enrichment broths, as well as growth promoting reagents like ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP). Enrichment broths operate with various selective systems to reduce growth

of accompanying flora and combinations of cefoperazone, vancomycin, polymyxin B, amphotericin B, colistin, trimethoprim and rifampicin are employed. Furthermore, culturing is performed at approx. 42°C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), adding to the selectivity (12, 13, 85).

Most media (broth and agar) for *Campylobacter* have been designed to support growth of *C. jejuni* and *C. coli*. As the genus comprises several potentially pathogenic species that are susceptible to the above-mentioned antibiotics, like *C. upsaliensis*, *C. lari*, *C. hyointestinalis* and *C. mucosalis*, this might lead to a misdiagnosis and underestimation of the burden of infection (120). Growth of *C. coli* has been shown to be inhibited by the selective agents included in Preston broth - but not in Bolton broth (Manuscript I). In contrast to these findings, Preston and Bolton broth supported the growth of thermotolerant *Campylobacter* from boot and cloacae swabs and chicken neck skin equally well (Manuscript IV). Other studies performed on the subject produced controversial results as well, probably due to differences in sample matrix, dominant *Campylobacter* species, and accompanying microflora. Bolton broth is currently recommended for enrichment in both the NMKL 119 and ISO 10272-1 standards, and it was also proven to be superior to Preston and Mueller-Hinton broth in supporting the growth of a test panel of *Campylobacter* strains of relevance to food safety (Manuscript I). For these reasons Bolton broth was chosen for enrichment of *Campylobacter* prior to PCR detection in Manuscript I. Applying filter techniques, like the Cape Town protocol, instead of selective antibiotics, is known to result in a significantly higher yield of species other than *C. jejuni* and *C. coli* (101). Some protocols e.g. BAM and Park-Sanders, recommend delayed addition of selective agents and/or a reduced initial incubation temperature to promote the recovery of injured cells and possibly also the growth of more sensitive species.

Following enrichment, or directly from samples with presumed high numbers of *Campylobacter*, the samples are spread on selective agar plates. Again a high number of solid media exist for *Campylobacter*, and modifications to existing selective agars are numerous as well. Some of the most common ones are: modified charcoal cefoperazone deoxycholate (mCCDA), Skirrow, Karmali, Preston, Abeyta-Hunt-Bark (AHB), Campy-cefex and Butzler. It is recommended to use two selective agars with different selective principles in parallel to increase the yield, but currently ISO 10272-1 is the only standard culture method prescribing this (12). Solid media for enumeration of *Campylobacter* should always be dried to avoid excessive moist and thereby obtain single colonies.

Identification of *Campylobacter* presumptive colonies is performed by subculturing five colonies from selective media onto non-selective media. These are examined microscopically regarding morphology and motility. Furthermore, a number of tests can be performed to confirm the identification and determine the species; growth at 25, 37 and 42°C, catalase, oxidase, glucose utilisation, hippurate hydrolysis etc. Besides the fact that *Campylobacter* are relatively biochemically inactive, former studies of their biochemical properties also reveal inconsistencies. In addition to this, biochemical identification can be encumbered with uncertainties derived from experimental variation, subjectivity of tests, anomalous properties of the single isolate and inconsistencies related to age and conditions of culture.

3.3 Limitations of culture-based detection

In summary, conventional culture methods for detection of *Salmonella* and *Campylobacter* are time-consuming and labour-intensive. A final positive test result can take up to 8 days. Furthermore, these methods are generally believed to suffer from poor specificity due to difficulties in recovering sublethally injured cells, problems in the identification of atypical colonies and a high degree of false-positive results. From a food safety point of view, these culture-based methods fail the purpose of producing a rapid and reliable result, which can be used pre-retail to ensure the microbiological safety of the products being released to the consumer. An important advantage of conventional culture methods is nevertheless that they produce an isolate that can be characterised further.

4. Detection by PCR

Since *Salmonella* and *Campylobacter* worldwide remain sources of human infection, causing serious illness and socioeconomic problems, a lot of effort has been devoted to the development and improvement of detection methods in clinical, environmental and especially food samples. PCR has been shown to be a valuable and advantageous alternative to culture-based detection regarding speed, limit of detection, selectivity, sensitivity and potential for automation (173).

4.1 The principle of PCR

PCR is the most widely used nucleic acid based technique today. In PCR repeated cycles of DNA synthesis is applied in order to replicate a target DNA sequence. In a few hours PCR amplification can produce millions of copies of a target DNA - in theory from a single copy - thus enabling the detection of DNA present initially in very low amounts (142). PCR is performed in a thermal cycling instrument and consists of three temperature steps, the first being heat-denaturation of the double stranded DNA performed at around 95°C. Following this, the temperature is lowered to 40-60°C, allowing the primers (short stretches of nucleotides designed to match the DNA target sequence) to anneal to the complementary nucleotides of the DNA target sequence. After the primers have annealed to the DNA target, the DNA polymerase enzyme initiates the extension of the primers by adding one appropriate nucleotide after another, creating a DNA strand complementary to the DNA target sequence (Figure 5). Extension usually takes place at 72°C to ensure optimal performance of the DNA polymerase enzyme. This three-stage cycle (denaturation, annealing and extension) is repeated 30-50 times in the thermal cycler, with the newly synthesised DNA strands acting as templates for the synthesis of new complementary DNA sequences. The rate of formation of target DNA sequences is thus exponential, and as a result the amount of target DNA is doubled in each three-staged temperature cycle. Conventional PCR employs end-point detection, where the PCR product i.e. the amplified DNA target sequence, is visualised by gel electrophoresis. The PCR products are loaded onto an agarose gel together with an appropriate molecular marker, eletrophoresis is performed and the gel is stained with ethidium bromide, and visualised by ultraviolet transillumination (59, 123, 142, 165). This technique was employed in Manuscript I and II.

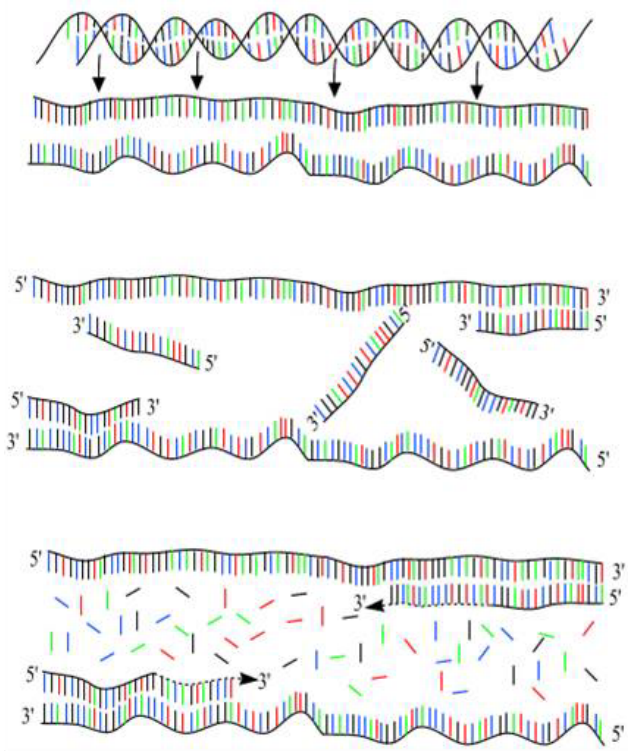


Figure 5. The principle of PCR showing the DNA denaturation and replication in the different temperature steps applied (25).

In real-time PCR the amplification can be monitored by incorporation of a fluorescently labelled probe or a DNA binding dye like SYBR green. A variety of probe types exist employing different principles (Manuscript VII), but all producing a change in fluorescence upon PCR product accumulation. This fluorescent signal is recorded after each temperature cycle, and the accumulation of PCR product thereby visualised. Real-time PCR is more sensitive than conventional PCR and it holds the possibility of quantification. Additional advantages compared to conventional PCR include: a reduced risk of cross-contamination from exposing the environment to the PCR products, a reduction in hands-on and total time of analysis and finally the possibility of a higher degree of automation (97, 110, 172). Real-time PCR techniques were applied in Manuscript III-VIII.

4.2 PCR inhibition

One of the drawbacks of applying PCR for detection of DNA is the sensitive nature of the method and the reagents which make it prone to inhibition. Inhibition can be exerted at several levels in PCR: 1) the availability of the DNA can be reduced due to inhibition of cell

lysis, 2) the DNA can be degraded or captured by inhibitors, and 3) the activity of the DNA polymerase enzyme can be reduced by inhibitors (186). Of these, the inhibition of the activity of DNA polymerase seems to be the most significant. This is also reflected in the fact, that inhibition in some cases can be overcome by simply replacing the DNA polymerase with one that exhibits more resistance towards the particular inhibitors (1, 95, 108). The *Tth* DNA polymerase, which has been applied throughout the present thesis (Manuscript I-VIII), has been shown to be resistant towards inhibition in a range of sample matrices (1, 92, 108, 109).

Several compounds, that can be found in samples, enrichment media and DNA extraction reagents, are known to inhibit PCR amplification e.g. lipids, proteins, blood, urea, ferric ammonium citrate, bile salts, esculin, acriflavin, magnesium chloride, detergents, lysozyme, NaOH, alcohols etc. (149, 186), (Manuscript I). Even though most of the inhibitory effects from sample and media constituents can be overcome by extracting the DNA from the sample prior to PCR amplification, it is an absolute prerequisite to include an internal amplification control (IAC) in PCR to avoid false-negative results due to technical problems with reagents or thermal cycler, or as a result of PCR inhibition (81). Without an IAC present in every single PCR well, a negative result should not be regarded as being true (78). Competitive IAC's are frequently applied, and the advantage of this approach is that multiplexing is avoided, as the IAC is amplified with the same primers as the target DNA. Using the competitive approach, it is critical that the concentration of the IAC is close to the limit of detection, in order not to extinguish the signal from the target DNA and to reveal even a slight inhibition of the PCR. Competitive IAC's are often designed larger in size than the target, to drive the reaction kinetics towards the target (39, 80). The IAC's for both the *Salmonella* and the *Campylobacter* real-time PCR were designed after this competitive strategy (Manuscript I-VIII).

Furthermore, it is important to include well-defined and appropriate test controls both during the development and validation, but also during implementation and use of a diagnostic PCR method. These include positive and negative processing controls to reveal errors in the different processing steps of the method (143). Finally, reagent control (non-template control, NTC), and positive and negative DNA controls should be included in each PCR run to verify that no PCR reagents are contaminated, and that the PCR responses are as expected.

4.3 Sample preparation

The differences in sample matrices, enrichment media and PCR targets demand careful consideration and optimisation of sample preparation in each single method. For this reason numerous sample preparation methods for PCR analysis have been published. No attempt to describe differences, advantages and limitations of various sample preparation methods will be attempted in this thesis (For a review see Rådström et al., 2004 (143)). Reducing the often large initial sample volume to a small homogeneous volume of purified DNA/RNA to be amplified in PCR is a challenge that has not yet been overcome. In most PCR methods DNA is extracted from a subset of the sample (usually < 5 ml) to avoid the inhibitory effects of other sample constituents, and typically 1-10 µl of this is added as template in the PCR. In the present thesis a simple resin-based extraction method (179) was found to overcome the inhibition from enrichment media and sample constituents in Manuscript I. This resin-based extraction method was applied both in the collaborative validation (Manuscript II), and in the development of the real-time PCR method (Manuscript III). Preliminary experiments conducted prior to the work described in Manuscript V, included a comparison study of several commercial manual DNA extraction kits, and several kits for the automated DNA extraction platform KingFisher (Thermo Labsystems, Helsinki, Finland). Applying the Magnesil KF, Genomic System (Promega Corporation, Madison, WI, USA) on the KingFisher platform for DNA extraction, was shown to be equal or superior to the other methods (data not shown). Other studies have shown the DNA recovery applying various extraction techniques to be strongly sample dependent (63, 155, 132). Since the methods developed and validated in Manuscripts III-VI were meant for use in routine laboratories with a high throughput and the need for a high degree of quality control, this automated DNA extraction procedure was applied throughout the remaining of this thesis.

The loss of DNA during extraction on the KingFisher platform was evaluated in Manuscript V, by comparing the results obtained in the real-time PCR method on samples containing a known amount of reference DNA, before and after DNA extraction. The experiment was designed in a way that theoretically equivalent amounts of DNA was analysed in PCR. The DNA loss was shown to be quite high (in average, results around 5 threshold cycle values higher were obtained post DNA extraction), especially in samples containing high levels of DNA, perhaps indicating a limitation in the binding capacity of the paramagnetic beads applied in the procedure. Increasing the amount of paramagnetic particles did however only lower the threshold cycle values slightly.

4.4 Strategies for optimisation of real-time PCR-based methods

Optimisation of sample preparation (Manuscript V), PCR master mix (Manuscript V), and using alternative probe chemistries (Manuscript VII) can be applied to optimise the overall performance of real-time PCR. The strategies described in the following sections are not unique to detection of *Salmonella* and *Campylobacter*, but could be applied to improve the sensitivity and optimise the performance of other real-time PCR-based methods as well.

The *Salmonella* method consists of three steps: Pre-enrichment, DNA extraction and real-time PCR analysis. In order to shorten the total time of analysis as much as possible, it was attempted to optimise all steps of the method (Manuscript V).

4.4.1 Optimisation of sample preparation

A reduction of the time of pre-enrichment, necessary for detection in real-time PCR, was attempted by evaluating the growth supporting capacity of different media. The effect of adding growth-promoting and background flora-reducing agents was investigated on a panel of *Salmonella* serovars of importance to meat. In accordance with the findings of other studies (66, 79, 175), no alternative media was identified as being significantly superior to BPW. The choice of BPW, which is recommended for pre-enrichment in both the ISO and the NMKL standard, also makes retrospective culture-confirmation of positive PCR results possible (Manuscript V).

Extraction of DNA from pre-enrichment volumes of 1, 2, 5 and 10 ml was performed (Manuscript V). Five ml was shown to improve the detection limit and produce steeper amplification curves. Even though it would be advantageous to operate with larger volumes, the available technology does not enable separation of the target from inhibitors and co-purified DNA from background flora, reducing the sensitivity of PCR (181).

The DNA extraction on KF is based on the binding of DNA to silica-coated paramagnetic beads under high concentrations of chaotropic salts, and the release of the collected DNA in a low-ionic strength buffer. Attempts to optimise the DNA extraction procedure was made by combining the different binding, washing and elution steps of the KF in various ways. The amount of paramagnetic beads was increased, which improved the PCR results, and the elution volume was decreased which gave poor and inconsistent PCR results (Manuscript V).

4.4.2 Optimisation of PCR master mix and probe chemistry

Optimisation of the PCR assay was performed by titration of the single components in the master mix, as well as the addition of the PCR adjuvants glycerol and DMSO to enhance the specificity and reduce formation of secondary structures (177). The thermal profile of the PCR assay was likewise optimised (Manuscript V).

The template volume for PCR was increased from 5 to 10 and 20 µl with lower threshold cycle values, steeper amplification curves and a higher reproducibility as a consequence (Manuscript V). However, this is not always the case as shown in Manuscript VIII and by Botteldoorn et al., 2008, where a clear PCR inhibition was observed when increasing the amount of template DNA in the PCR (35).

In Manuscript VII it was shown that optimising the probe chemistry can be a convenient way to improve the sensitivity of a real-time PCR assay. The performance of the three probe chemistries: locked nucleic acid (LNA) (36), minor groove binder (MGB) (3) and Scorpion (uni- and bi-molecular) (185) were compared to a conventional TaqMan probe.

A real-time PCR probe should be designed to obtain a melting temperature around 5-10°C higher than the corresponding primers, in order to ensure correct cleavage of the probe element during extension. For conventional TaqMan probes, this often results in 25-30 nucleotides long probes, which can be difficult to design in case of a short specific region.

Claimed advantages of the short probe types like LNA and MGB are improved sensitivity towards single-nucleotide mismatches, and improved signal-to-noise ratio due to reduced spurious annealing and a reduced distance between the reporter fluorophore and the quencher (98).

While conventional TaqMan, LNA and MGB probes are hydrolysis probes, with the reporting fluorophore (R) separated from the quencher (Q) by the 5'-3'-exonuclease activity of the DNA polymerase during the amplification process (Figure 6), the Scorpion system relies on an unique intra-molecular probing mechanism as shown in Figure 7. Theoretically, the Scorpion chemistry should be more sensitive than the other probes evaluated in Manuscript VII, due to this intra molecular probing mechanism. In addition the quenching mechanism is collisional rather than relying on through-space quenching like TaqMan, LNA and MGB probes (161, 166). The Scorpion chemistry was, however, not compatible with the real-time PCR assay in the present thesis (Manuscript VII).

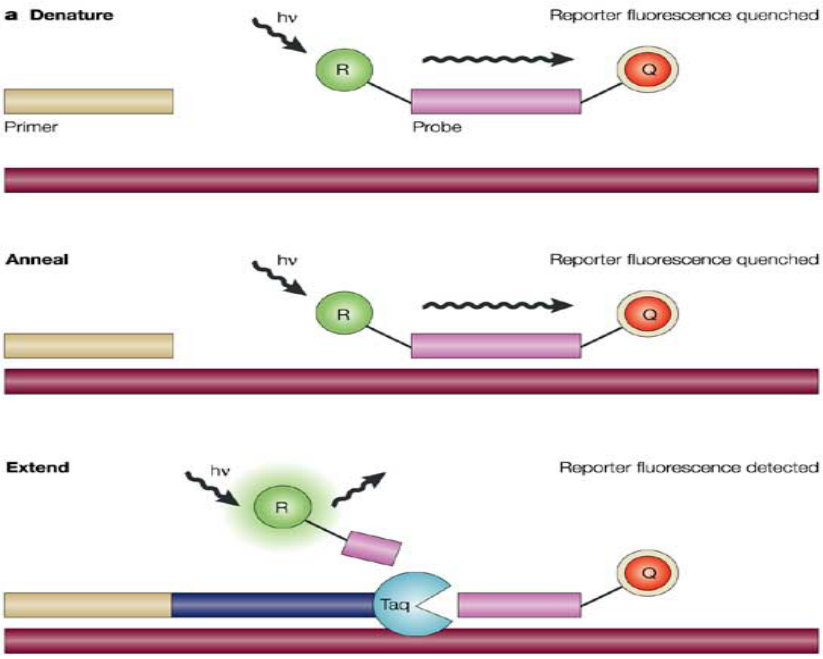


Figure 6. Principle of a hydrolysis probe. The probe is labelled with a reporting fluorophore (R) in one end and a quencher (Q) in the other. The proximity of the quencher to the reporter while the probe is intact, suppresses the signal from the reporter. Upon extension of the primer by the DNA polymerase, the probe is hydrolysed, the reporter separated from the quencher, and the fluorescence detected. Reprinted with permission from Koch (2004) (96).

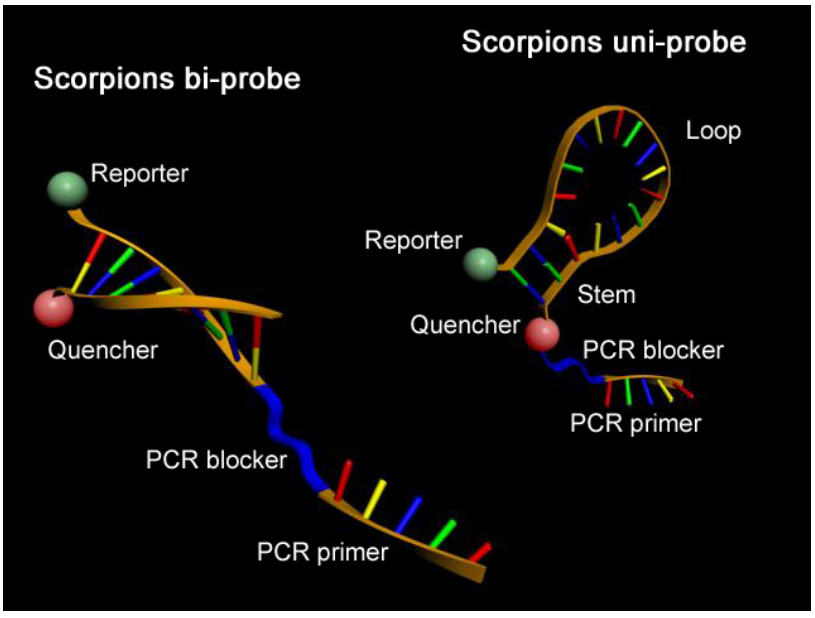


Figure 7. The structure of uni- and bi-molecular Scorpion probes. The uni-molecular system consists of a probe element, inserted in a molecular beacon configuration, labelled at the ends with a quencher and a fluorophore, respectively. The tail is linked to the 5'-end of a primer via a PCR-blocker. Upon annealing and extension of the primer a complementary probe site is created. Following the next denaturation cycle, the probe element will fold up and anneal to this, rather than entering the hairpin loop conformation again, because it is kinetically favourable. The reporter is thus separated from the quencher, and fluorescence can be detected. The bi-molecular system operates after the same principle, only the hairpin loop configuration is replaced by two probes, one labelled with a reporter and one with a quencher. The bi-molecular system is an improvement of the technology, and should give rise to an increased fluorescent signal, since the distance between the reporter and the quencher is increased compared to the uni-molecular type where they remain on the same DNA-strand (19, 26, 185). Reprinted from (26).

The results obtained in this comparative study proved the LNA probe to be the most sensitive probe chemistry, producing the lowest threshold cycle values and the highest amplification efficiency. Inconsistent results have been obtained in other studies comparing some of the same probe chemistries (46, 105, 145), emphasising the need to individually evaluate the optimal probe chemistry for a given PCR assay, as the conclusion drawn from one comparison study not necessarily is generally applicable.

4.5 Validation for routine diagnosis

Despite the abundance of PCR methods for detection of foodborne pathogens, a lack of proper method validation has hampered the dissemination and implementation of PCR in diagnostic routine laboratories (78, 115).

The sensitive nature of the PCR technique can be an impediment for implementation, as the performance of a PCR method can vary significantly depending on the sample matrix, sample preparation (Manuscript I), PCR reagents (38, 189), thermal cycler (145) (Manuscript III) and personnel. The results of a PCR method developed in one laboratory can be very difficult to reproduce in another. A diagnostic PCR method, to be used as a tool to ensure food safety and public health, has to be reliable and consistent, day after day, in the hands of different personnel, on different sample matrices and different thermal cyclers. For these reasons, validation following an integrated approach including sample matrix, sampling, sample treatment and PCR is necessary.

The basic objective of validating a PCR-based method for foodborne pathogens is to demonstrate that it can produce results that are comparable or superior to the current standard method. Several validation organisations like ISO, Deutsches Institute für Normung (DIN), Association Française de Normalisation (AFNOR) and NordVal have issued protocols and guidelines for the validation of alternative microbiological methods. In the present thesis, the real-time PCR-based methods for detection of *Salmonella* and *Campylobacter* were validated according to the NordVal protocol, and subsequently approved (Manuscript IV and VI), (27, 28). The overall flow of the validation procedure for both of the real-time PCR-based methods, showing the contents and results of the comparative in-house study and the collaborative trial is shown in Figure 8.

The *Salmonella* method has been implemented for screening of samples at major Danish slaughterhouses, reducing the post-slaughter storage time and facilitating the fast release and export of *Salmonella*-free fresh meat (Manuscript VI). The *Campylobacter* method was implemented at one of the large Danish poultry slaughterhouses, and used for logistic slaughter planning, as well as for production of certified *Campylobacter*-free chickens (Manuscript IV).

Retrospectively, the collaborative trials performed in this study have limitations. The first trial conducted, to validate the conventional PCR method for *Campylobacter*, lacked the appropriate controls to reveal possible cross-contamination in the participating laboratories. The ring-trial performed on *Salmonella* real-time PCR was restricted to one brand of thermal cyclers, on which the method had also been developed and optimised. It was later shown that the reproducibility of the method using other thermal cyclers was poor (data not shown). The comprehensive optimisation of the method using only this brand of thermal cycler, to achieve a lower limit of detection, and thereby being able to shorten the pre-enrichment time as much as possible, resulted in a loss of robustness. Finally, the pre-enrichment step of the method was not performed by the ring-trial participants, but at the organising laboratory. This had been approved by NordVal in advance, and the approach was chosen to minimize variations not attributable to the molecular based detection step. Future studies need to take these experiences into consideration, and adjust their procedures accorcingly.

4.6 PCR detection of *Salmonella*

Numerous PCR assays detecting *Salmonella* in a variety of sample matrices have been described in the literature. The majority of them deal with detection in food, reflecting the epidemiology of this human pathogen. The PCR-based detection methods in food commonly employ a pre-enrichment step combined with subsequent PCR detection. The pre-enrichment times reported vary from 6 to 24 hours, depending on the artificial inoculation level of *Salmonella* in the experimental design of the studies (Manuscript V), (4, 58, 60, 74, 114, 124). Studies taking the limit of detection of 1 CFU/25 g in food samples into consideration, report a minimum of pre-enrichment of 8-10 hours, as in the present thesis (Manuscript V and VI), (43, 67, 74). Several of these PCR assays amplify a part of the *invA* gene, encoding a protein involved in the invasion of epithelial cells, however, it has been shown that *invA* is lacking in some strains (69, 144).

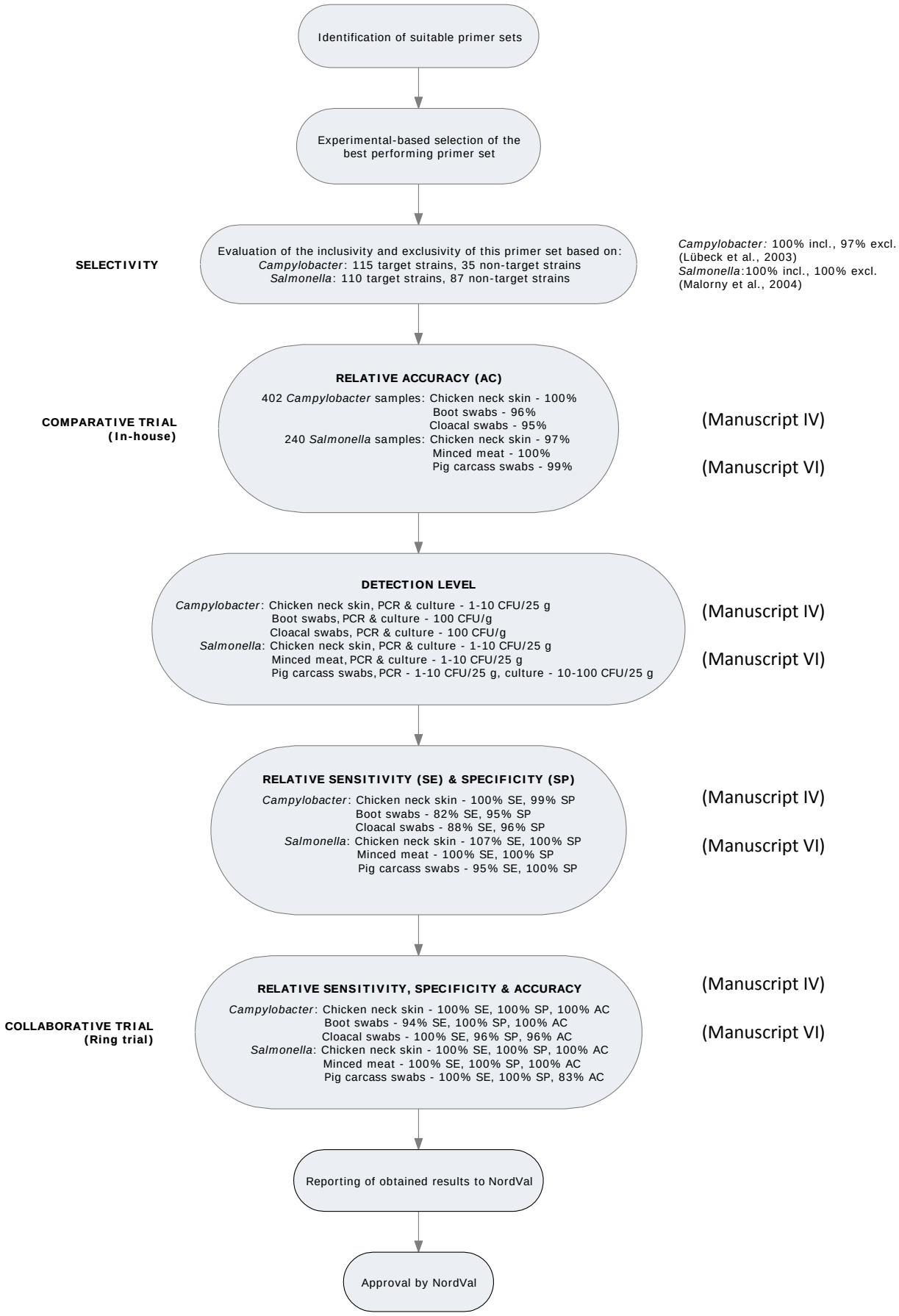


Figure 8. The general steps of the validation procedure (10).

Malorny et al. (2004) recently designed a PCR assay for amplification of a part of the *ttrRSBCA* locus encoding proteins used for tetrathionate respiration (114). The inclusivity and exclusivity of this assay was tested applying 110 *Salmonella* strains and 87 non-*Salmonella* strains, and was found to be 100 % in both cases. The assay has furthermore been validated in collaborative trials and found to perform satisfactory (116). This PCR assay forms the basis for the *Salmonella* detection method developed and validated in Manuscript V and VI. Following substantial optimisations (Manuscript V), the final real-time PCR-based method was able to detect *Salmonella* in minced meat and chicken skin samples after 12 ± 2 hours of enrichment and in pig carcass swab samples after 14 ± 1.5 hours of enrichment (Manuscript VI), compared to at least 3 days for culture-based detection (18). The analysis time of this non-commercial, open-formula PCR-based method is comparable with the fastest validated DNA-based analysis kit (Bio-Rad, GeneSystems) for meat samples, and 1-3 hours shorter for pig carcass swab samples.

PCR detection methods have also been described for human clinical samples. A number of them are designed to target specific serovars and have little use as diagnostic screening tools, but more broad methods for *Salmonella enterica* have compared favourably to conventional culture-based methods in several studies (6, 156).

Commercial systems based on the PCR detection principle are available for *Salmonella* (TaqMan® *Salmonella* kit from Applied Biosystems, iQ-Check™ *Salmonella* kit from Bio-Rad). One of the most widely used is the BAX® (DuPont Qualicon, Wilmington, Delaware), an integrated system where pre-enrichment of 22-26 hours, followed by 3 hours of regrowth depending on the sample matrix, is followed by DNA extraction and automated PCR (30, 31, 51). The BAX system has been validated against traditional culture methods and is currently approved by several standardisation organisations for *Salmonella* testing in a variety of foods (22). The real-time PCR based method developed and validated in the present thesis was also compared successfully to the BAX system for detection of *Salmonella* in artificially contaminated pork filet samples (Manuscript VI).

Quantitative real-time PCR methods for *Salmonella* will not be discussed in this thesis (for a review see (113)).

4.7 PCR detection of *Campylobacter*

Numerous conventional, gel-based PCR methods for detection of *Campylobacter* have been published, targeting a variety of genes and species, and tested on many different sample types. Many of the methods are developed for detection in chicken and chicken products, reflecting the importance of this as a source of human *Campylobacter* infections (56, 112, 122, 191). A majority of PCR primers applied for detection of *Campylobacter* target the 16S rRNA gene as in the present thesis, while species specific assays are often designed to recognise the *mapA* or *hipO* gene for *C. jejuni* and the *ceuE* gene for *C. coli*.

For PCR detection of *Campylobacter* in food, where a relatively low amount of target bacteria is suspected, an enrichment step prior to PCR is often applied. Though adding to the total time of analysis, several advantages are obtained by this: the PCR inhibitory sample constituents are diluted, the level of *Campylobacter* is increased to allow detection and the ratio between live and dead cells is increased substantially, making the contribution to PCR response from dead cells negligible. Furthermore, in order to meet the legislative demands of detection of 1 CFU/25 g (12), the current techniques available does not leave any option but enrichment of samples with a low level of *Campylobacter*.

Manuscript I and II, describes the development and validation of a conventional PCR-based method for detection of *Campylobacter* in chicken carcass rinse and pig swab samples where an enrichment step was employed. This method was shown to have a sensitivity and specificity similar to the current standard culture method (15) both in-house (Manuscript I) and in a multicenter collaborative trial (Manuscript II). Since an internal amplification control (IAC) was also included, the method was found suitable for routine diagnosis. The total time of analysis for negative samples was 2 days for the PCR-based method, compared to 5 days for the culture-based method.

Commercial systems for PCR-detection of *Campylobacter* likewise employ enrichment steps of 24-48 h for samples with low contamination levels (iQ-Check™ *Campylobacter* kit from Bio-Rad, BAX® from DuPont Qualicon, and TaqMan® *Campylobacter jejuni* detection kit from Applied Biosystems). The BAX system has obtained AOAC performance approval for qualitative detection of *C. jejuni, C. coli* and *C. lari* in ready-to-eat turkey products and chicken carcass rinse samples.

Methods for detection, differentiation and quantification of *Campylobacter* spp. in clinical samples have been shown to be more sensitive and reliable, as well as work- and time-saving compared to culture methods (99, 106).

PCR assays for post culture identification and differentiation of *Campylobacter* colonies are increasingly replacing the uncertain biochemical methods described in Section 3.2. Debruyne et al., (2008) presents a comparison study of available assays (55).

5. Quantification of *Campylobacter*

5.1 Quantitative real-time PCR

Several real-time PCR methods for *Campylobacter* have been described in the literature, albeit most of them concern detection and quantification ***after*** enrichment which is a contradiction in terms (2, 133, 140, 154). As shown in Manuscript III, it is possible to establish a linear correlation between the threshold cycle values obtained in real-time PCR and the CFU in enriched samples, but as means of quantification of the original number of *Campylobacter* cells present in a given sample, such a method is encumbered with uncertainties.

Direct quantitative real-time PCR methods for *Campylobacter jejuni* and *C. coli* in poultry have been published (Table 1) (35, 53, 77, 131, 147, 191). Although, bearing in mind that several of the current control strategies regarding *Campylobacter* are focused on post slaughter reduction of the number of bacterial cells on the chicken carcass, the usefulness of these real-time PCR methods for quantification could be limited, since they detect all *Campylobacter* present in a sample, including the dead cells. Furthermore, none of the published methods have been validated, the specificity of the primer sets has not been established according to the current standards, and none of them include an IAC. Another drawback of the majority of these methods is that the standard curves applied to quantify *Campylobacter* was made from serial dilutions of extracted DNA, not taking into account the fact that the efficiency of DNA extraction can vary substantially with the initial amount of cells (Manuscript V), (44). Finally, the natural variation attributable to the matrix in which the quantification was performed was not thoroughly investigated in any of the methods. This is a prerequisite for successful quantification that would be flawed and encumbered with uncertainties if large natural matrix variation was observed. The variation of the chicken rinse matrix, for which the present method was developed, was evaluated and found to be negligible (Manuscript VIII).

Since the *Campylobacter* real-time PCR method in the present thesis quantifies *C. jejuni*, *C. coli* and *C. lari* simultaneously, without differentiation, equal sensitivity and amplification efficiency for all three species has to be assumed. To test this hypothesis, the variation attributable to individual species was investigated on both DNA and cell level. The variance between the three species was not significant on DNA or cell level, proving equal sensitivity

Table 1. Performance characteristics of the quantitative real-time PCR methods for *Campylobacter*.

Target	Linear range	R^2	Efficiency (%)	Limit of detection	Differentiation of viable/dead	IAC	Reference
Campylobacter spp.	3.8-3.8×10^7 DNA copies[a]	0.99	97	7 CFU/ml	No	No	(35)
C. jejuni	1.0-1.0×10^4 CFU/ml[a]	0.98	96	6-15 CFU/PCR	No	No	(191)
C. jejuni	1.0×10^1-1.0×10^6 CFU/ml[a]	-	96	10 CFU/ml	No	No	(147)
C. jejuni and *C. coli*[b]	1.0×10^1-1.0×10^7 CFU/ml	0.99	86	10 CFU/ml	No	No	(77)
C. jejuni	1.0-1.0×10^6 CFU/ml[a]	-	96	1 CFU/ml	No	No	(53)
C. jejuni	1.0×10^1-1.0×10^6 CFU/ml	1.00	88	10 CFU/PCR	No	No	(131)
C. jejuni, *C. coli* and *C. lari*	1.0×10^2-1.0×10^7 CFU/ml	0.99	91	10 CFU/PCR	Yes	Yes	Manuscript VIII

[a] Standard made from serial diluted pure DNA

[b] differentiation and quantification by duplex PCR

and amplification efficiency of the method independent of species (Manuscript VIII). The standard curve applied for absolute quantification was therefore made from *C. jejuni* CCUG 11284 in *Campylobacter*-free chicken carcass rinse.

In order to perform correct quantification it is essential that a PCR assay performs with relatively constant amplification efficiency and has a linear range of relevance to the actual sample-response. The linearity of the real-time PCR method was evaluated both on pure DNA in Manuscript III and on cells in Manuscript VIII. In Manuscript III, the number of genomic DNA copies was plotted against the threshold cycle value obtained, and a linear relationship could be established. The number of genomic copies was determined using the equation $m = n(1.013 \times 10^{-21})$, where m is the mass, and n the number of base pairs in the genome, determined to be 1,641,481 in *C. jejuni* NCTC 11168 (139). The linear range was between 5×10^{1} and 1×10^{7} copies of DNA on the RotorGene instrument, and between 1×10^{3} and 1×10^{7} copies of DNA on the ABI-PRISM 7700 (Manuscript III). In Manuscript VIII, the linear range of the method was determined to be 1×10^{2} to 1×10^{7} CFU/ml chicken carcass rinse, and the limit of quantification thereby 1×10^{2} CFU/ml, corresponding to 10 CFU/PCR (Table 1).

The amplification efficiency (AE) was calculated in Manuscript III and VIII using the slope of these linear relationships by the equation $AE = 10^{-1/slope}-1$ (94), and was determined to be approx. 90% independent of the thermal cycler (RotorGene 3000, ABI-PRISM 7700, Mx3005P), reflecting the robustness of the assay.

Alternative sample preparation methods for quantitative detection of *Campylobacter* by PCR have been published. Wolffs et al. (2005) described the use of flotation prior to real-time PCR to quantify viable and VBNC *Campylobacter* (190). The quantification was performed by applying a discontinuous buoyant density gradient method to separate viable and VBNC cells from the sample matrix and background flora, and amplifying only this cell fraction in PCR. The limit of detection of this flotation-based method was determined to be 8.6×10^{2} CFU/ml and quantification was possible in the range of 2.6×10^{7} down to 2.6×10^{3} CFU/ml in chicken rinse samples.

The feasibility of large-volume immunocapture of *Campylobacter* prior to PCR, reducing sample volumes from 250 ml to only 200 µl has also been evaluated (121). The cell capture and recovery of *Campylobacter* was shown to be quite low, and the method only suitable for enriched samples with high levels of target cells.

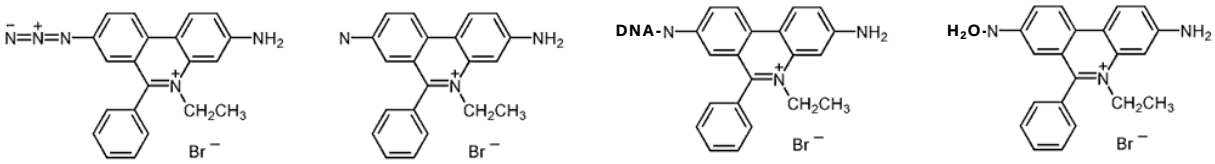
5.2 Differentiation of viable and dead *Campylobacter*

As many advantages as the real-time PCR technology offers regarding pathogen detection in mixed populations, a major drawback has been that PCR is not able to distinguish between DNA from viable and dead cells. DNA from dead cells has been shown to persist for up to three weeks following cell death (90). This has been one of the main causes hindering the implementation of PCR in routine diagnostics for food, where quantification of viable bacteria is essential. It is a serious limitation of the PCR technology, which is of particular relevance for *Campylobacter*, since mitigation strategies are focusing increasingly on post slaughter decontamination, leaving the PCR technology unable to assess the potential for foodborne infections. The presence of DNA from dead *Campylobacter* can lead to an overestimation of the number of viable cells, and in some instances even to a false positive response (188).

Through the last decades lots of effort has been devoted to develop techniques that allow distinguishing between viable and dead bacteria in mixed populations. However, no ultimate method has been identified, as they all suffer from serious drawbacks (42). Conventional culture to characterise viability of bacteria is time consuming, and it is known that some strains of *Campylobacter jejuni* can enter a viable but non-culturable (VBNC) state following unfavourable conditions, which is not detected by conventional culture (146). Flow cytometry methods can differentiate between viable and dead bacteria, but are not species specific, and can therefore only be applied in combination with other techniques (37). Specific amplification of mRNA by reverse transcriptase PCR or nucleic acid sequence-based amplification (NASBA) has been applied extensively as a viability marker due to mRNAs intrinsic instability (42). This approach is likewise problematic, since the expression and transcription is relatively unstable, and some mRNA molecules are not transcribed in the VBNC state. Furthermore, using mRNA as a viability marker, several parameters such as physiological cell condition, lethal treatment and period of time after treatment until analysis, extraction and analysis method can influence the result (91). For *C. jejuni* it has been shown that both lethal treatment and post-treatment holding conditions had a marked influence on the results obtained using mRNA as a viability marker (153).

A novel concept for distinguishing between DNA from viable and dead cells employing a pre-PCR ethidium monoazide (EMA) sample treatment was described by Nogva et al. (2003) and Rudi et al. (2005), (130, 151). EMA ($C_{21}H1_8BrN_5$; phenanthridium, 3-amino-8-azido-5-ethyl-6-phenyl bromide, Figure 9) can intercalate into the double helical DNA, and upon exposure to intense visible light the ends of the EMA molecule will cross-link the two DNA strands (76), leaving it unavailable for PCR amplification. The intensive visible light exposure will

simultaneously convert the free EMA in the sample to hydroxylamine that will no longer cross-link DNA (Figure 9).



A. Ethidium monoazide B. Nitrene C. Cross-link with DNA D. Hydroxylamino ethidium

Figure 9. Structural changes of EMA following photoactivation. A: Ethidium monoazide in its native structure. B: The azide group is converted to nitrene following visible light exposure. C: The DNA intercalated EMA binds covalently to DNA via the nitrene following light exposure. D: The unbound EMA reacts with water upon light exposure forming hydroxylamino ethidium.

Viable cells with intact cell membranes have been shown to be relatively impermeable to EMA, hence treating a sample with EMA followed by photolysis will result in a real-time PCR signal from only viable bacteria. The principle of EMA-PCR is shown in Figure 10.

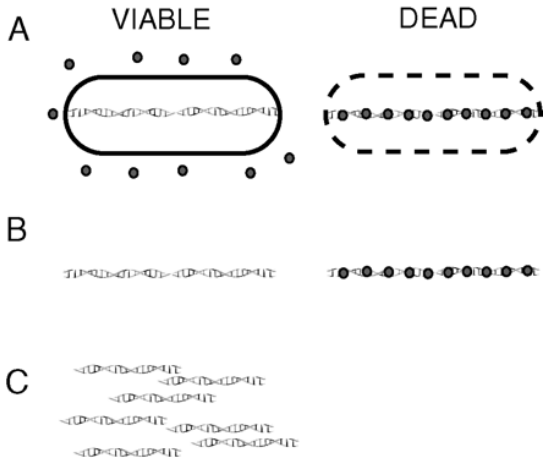


Figure 10. The principle of EMA-PCR. Adapted with permission from Rudi et al., 2005 (151). A) EMA is added to a sample pre-PCR and during a short incubation it intercalates into the accessible double helical DNA of cells with permeable membranes. The sample is exposed to intensive visible light for a fixed period of time, and the two DNA strands are cross-linked irreversibly by EMA, while the unbound EMA is converted into hydroxylamine. B) After DNA extraction two fractions of DNA are present; the EMA cross-linked arising from dead cells and the unstained fraction arising from viable cells. C) The unstained DNA fraction will be denatured and amplified in the PCR, while the cross-linked DNA will not be available for PCR amplification.

The principle of EMA-PCR has however been questioned in a study by Nocker and Camper (2006), where it was suggested that the reduced PCR signal from EMA treated dead bacterial cells, to a considerable extend, was due to loss of EMA-bound DNA during DNA extraction, rather than inhibition of amplification (128). Their findings suggest that the EMA-DNA conjugates from dead cells are cross-linked to other cell components and pelleted together

with cell debris during the centrifugation step applied in the DNA extraction. This theory was disputed by Hein et al. (2006), as being unsubstantiated (75), and the general understanding of the mechanism remains as shown in Figure 10.

EMA has been applied successfully in several studies to differentiate between viable and dead *Klebsiella oxytoca*, *Escherichia coli* O157:H7, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Listeria monocytogenes* and *Vibrio vulnificus* (128-130, 159, 160, 182). Furthermore, it has been shown to selectively allow a PCR signal from viable *L. monocytogenes* on cheese and in milk, *C. jejuni* on chicken meat, and viable microflora from fish fillets (103, 104, 150, 151, 159).

In later studies, EMA has been reported to penetrate the cell membrane of intact viable cells. EMA concentrations of 10 and 100 μg/ml killed all viable *Anoxybacillus flavithermus* in milk powders, while concentrations of 0.1 and 1 μg/ml resulted in survival rates of 58% and 0.75% (152). Also for *L. monocytogenes*, *E. coli* O157:H7, *Micrococcus luteus*, *Mycobacterium avium*, *Pseudomonas syringae*, *Staphylococcus aureus* and *Enterobacter sakazakii* EMA treatment resulted in a loss of genomic DNA from viable cells (41, 129, 135). The viability of these bacteria was compromised, in varying degrees, and the conclusion drawn from these studies was that viable cells are not exclusively impermeable to EMA. Since the permeability differs with the particular bacterial species, the agent can not be regarded as generally membrane impermeable, and therefore real-time PCR analysis of EMA-treated samples will lead to unpredictable underestimations of the number of viable cells.

In 2006, Nocker et al. published a study where EMA was replaced by propidium monoazide (PMA, $C27H33Cl2N_6$, phenanthridium, 3-amino-8-azido-5[3-(diethylmethylammonio)propyl]-6-phenyl dichloride) to differentiate between viable and dead bacteria by selective removal of DNA from dead cells (129). PMA was found exclusively in cells with compromised membranes, and it was demonstrated that no uptake occurred in cells with intact cell walls. The reason for this enhanced impermeability has been ascribed to the higher charge of the PMA molecule, which has two positive charges compared to only one for EMA. PMA is a chemical alteration (additional azide group) of propidium iodide (PI), one of the most frequently applied membrane-impermeable dyes in flow cytometry, and can be expected to have the same permeability potential as PI. This is of great value in a food safety perspective, since PI only penetrates permeabilised cells, and not the reproductively viable, metabolically active or cells with intact membranes, which can be resuscitated and therefore hold the potential of infection (126).

5.3 Post-slaughter quantification of viable *Campylobacter* on chickens

Real-time PMA-PCR has been successfully applied for quantification of viable *L. monocytogenes* (135) and *E. coli* O157:H7 (129), however these experiments were performed using laboratory cultured strains, not on naturally infected samples with the bacteria imbedded in a food matrix. In the present thesis, a PMA sample treatment was applied prior to direct quantitative real-time PCR on *Campylobacter* in chicken carcass rinse from 50 naturally infected chickens from a *Campylobacter*-positive flock (Manuscript VIII).

The chickens were collected at the abattoir immediately after cooling, and the number of *Campylobacter* in the chicken carcass rinse samples was determined in parallel by real-time PCR (with and without PMA sample-treatment) and conventional culture (13). The standards (Figure 11) were included in every PCR analysis and the quantity of *Campylobacter* in the naturally infected chickens was determined by extrapolation from the threshold cycle value obtained. Out of the 50 chickens, 42 were found *Campylobacter*-positive by culture, 45 by real-time PCR with PMA-treatment, and 48 by real-time PCR without PMA-treatment. The culture-negative/Q-PCR-positive chickens were shown to harbour low levels of *Campylobacter*, below the quantification limit of the Q-PCR. The same applied for chickens found *Campylobacter*-positive by Q-PCR without PMA-treatment, but *Campylobacter*-negative with PMA-treatment. The level of infection in the *Campylobacter*-positive chicken carcass rinses ranged from 25 to 1.5×10^6 CFU/ml, reflecting the post-slaughter difference in contamination within a flock.

The results obtained clearly showed a reduced signal in real-time PCR from the samples that had received PMA-treatment prior to PCR compared to the untreated parallel samples, indicating presence of DNA from dead *Campylobacter* cells. The signal-reduction was positively correlated to the level of *Campylobacter* in the samples, and a range of reduction from 1×10^2 to 2.4×10^6 *Campylobacter* cell equivalents (CCE)/ml was observed.

The PMA-PCR quantification compared favourably to direct culture-based detection of *Campylobacter*. Analysis of variance comparing Q-PCR (with and without PMA) to plate counts on mCCDA, showed that Q-PCR with PMA-treatment produced results that were statistically significantly closer to counts on mCCDA ($P < 0.001$) than Q-PCR without PMA-treatment. The relative specificity of the PMA-PCR method was 100%, and it was shown to be more sensitive than the culture-based method (Manuscript VIII).

The results obtained from PMA-PCR corresponded well to those obtained by culture, suggesting that VBNC *Campylobacter* were not present in great numbers in the samples.

However, these chickens were collected immediately after cooling at the abattoir and the ratio between viable and VBNC cells can only be expected to decrease as a consequence of food processing and storage, thus presenting a possible diagnostic uncertainty. Furthermore, post slaughter mitigation strategies to reduce the number of viable *Campylobacter* on chicken carcasses, will most certainly contribute to the number of VBNC cells. As long as the infectious potential of the *Campylobacter* VBNC state is not clarified, quantitative methods for risk assessment should detect these as well.

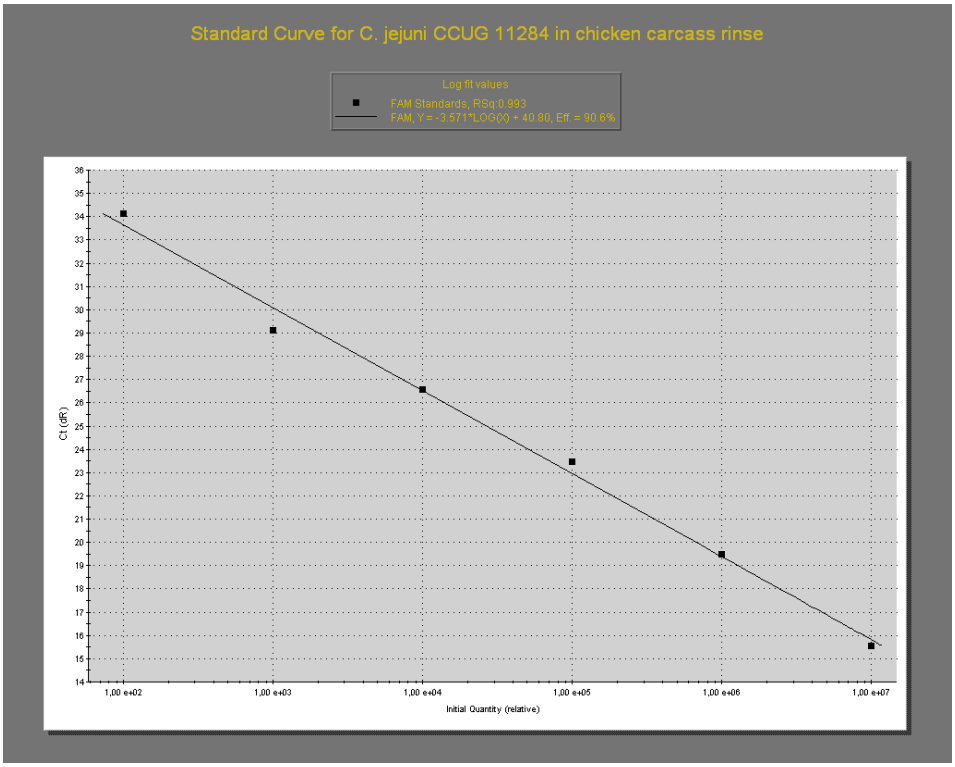


Figure 11. Standard curve applied for absolute quantification of *Campylobacter* in naturally infected chickens, showing the initial quantity on the x-axis and the corresponding threshold cycle value on the y-axis. The standard was prepared by duplicate PCR analyses of DNA extracted from 1 ml chicken carcass rinse inoculated with 1×10^2 to 1×10^7 CFU. The standard was included in every PCR analysis, and the quantity of *Campylobacter* in the naturally infected chickens was determined by extrapolating a quantity from the threshold cycle value obtained.

It has been demonstrated in several studies comparing Q-PCR to culture-based enumeration, that higher counts were produced by Q-PCR, which has been explained by the detection of DNA from dead and VBNC cells (35, 77, 191). In the present thesis, this was also true for the PMA-untreated samples. However, when the PMA-treatment was applied a slightly lower PCR quantification was often observed (Manuscript VIII). The reason for this could partly be due to underestimation of the cell input in the standard applied for quantification, but also due to overestimation of the number of *Campylobacter* colonies on the mCCDA plates.

Verification of colonies from the selective plates did indicate that not all *Campylobacter*-like colonies could in fact be confirmed as being *C. jejuni*, *C. coli* or *C. lari*, substantiating this theory. Another issue that has to be considered in this regard, is that the cell state and permeability of the cell wall is not a clear cut reflection of a viable or dead cell, and PMA could have entered a minor fraction of culturable cells.

The quantification limit of the present PMA-PCR does not meet the legislative demands of detection of 1 CFU/25 g (12). No available technology can enable direct PCR detection of 1 *Campylobacter* in 25 g. Adding to this dilemma, preliminary experiments also showed that a rinsing volume of 50 ml, far from recovered all the *Campylobacter* cells present on a chicken carcass. However, a rinsing volume of 310 ml was applied in a study by Jørgensen et al. (2002), and similar recovery rates to the present thesis was found (89). This factor has to be considered in estimating the level of infection and whole carcass contamination. Direct Q-PCR methods for *Campylobacter* are albeit applicable as tools for risk assessment and assurance of food safety, since it has been shown that a strong positive correlation exists between the number of *Campylobacter* on chickens and the risk of human infections (118, 148, 163).

6. Concluding remarks

The incidence and significance of foodborne diseases is escalating worldwide. The globalisation of the food supply, changes in the food production system as well as in the human and microbiological populations has led to an increased frequency of infection. In order to prevent and control two of the most important foodborne pathogens i.e. *Salmonella* and *Campylobacter*, improved detection methods are imperative. Rapid and reliable methods that can quantify and discriminate between viable and dead bacteria present in the food chain, can contribute to our knowledge on the fate of bacteria throughout the production chain, of transmission routes, persistence and prevalence, hence forming the basis for development of intervention and elimination strategies.

Even though the PCR technology offers valuable advantages compared to culture-based standard methods, the implementation of this technique has made slow progress. A lack of validated standard methods has made reproducibility of results difficult, and the fact that PCR response from dead cells could obscure the diagnostic outcome of the test has also been an impediment.

The focus of the present thesis has been to improve molecular-based methods for detection and quantification of *Salmonella* and *Campylobacter*. The thesis has described the development and validation of qualitative and quantitative PCR-based methods for these pathogens. The quantitative real-time PCR-method for *Campylobacter* was furthermore combined with a PMA-treatment to enable detection of viable target cells only. This method presents a cost-effective diagnostic tool ready to apply in industry and control laboratories, not detecting DNA from dead *Campylobacter*, but recognising the infectious potential of the VBNC state, and thereby able to assess the outcome and impact of new mitigation strategies.

The methods presented in this thesis could advantageously replace culture-based detection and quantification methods, and thereby reduce the time and cost of standard food analysis for *Salmonella* and *Campylobacter*. The rapid screening for negative, and identification of positive, samples, would contribute to food safety, assessment and prolongation of shelf life and, in addition, liberate resources to focus on suspected positive samples regarding typing and source tracing. The methods are particularly suitable for studying the fate of these bacteria throughout the process chain from farm to fork, in elucidating the effect of process steps in terms of reducing, eliminating or inactivating food pathogens introduced at various

steps. Thus, the methods can be used for risk assessment and evaluation of risk mitigating interventions.

In conclusion, this thesis will contribute to the dissemination of PCR-based detection methods for *Salmonella* and *Campylobacter*.

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Towards an international standard for PCR-based detection of foodborne thermotolerant campylobacters: interaction of enrichment media and pre-PCR treatment on carcass rinse samples

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Abstract

As part of a large EU project for standardisation of polymerase chain reaction (PCR), a systematic evaluation of the interaction of enrichment media, type of DNA polymerase and pre-PCR sample treatment for a PCR detecting thermotolerant campylobacters was carried out. The growth-supporting capacity and PCR compatibility of enrichment in Preston, Mueller–Hinton and Bolton broth (blood-containing and blood-free) were evaluated. The effect of resin-based DNA extraction and DNA extraction by boiling on the final PCR assay was investigated. The time-course studies indicated that a 20-h sample enrichment in blood-containing Bolton broth, followed by a simple resin-based extraction of DNA and a PCR amplification using T*th* polymerase, resulted in strong and clear PCR amplicons for target (287 bp) and internal amplification control (IAC, 124 bp). The enrichment PCR-based method, tested on 68 presumably naturally contaminated poultry-rinse samples, showed a diagnostic sensitivity of 97.5% (39 PCR-positive/40 total positive samples) and a diagnostic specificity of 100% (28 PCR-negative/28 total negative samples; $P = 0.32$) when compared to a standard bacteriological method (ISO 10272).

Keywords: *Campylobacter*; PCR; Enrichment; Carcass rinse; Food

1. Introduction

Thermotolerant *Campylobacter*, particularly *Campylobacter jejuni* and *Campylobacter coli*, are presently the most common causes of human foodborne infections in several developed countries where the number of reported cases of campylobacteriosis by far has exceeded the number of cases of salmonellosis (Altekruse et al., 1999; Friedman et al., 2000). Despite a low fatality rate associated with campylobacteriosis, the personal distress and the economic impact in form of medical visits, medication, hospitalisation, and loss of productivity is extensive (Friedman et al., 2000). This emphasises the importance of efficient and reliable detection methods in food and in the food production chain.

Several non-commercial and open-formula polymerase chain reaction (PCR)-based detection meth-

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ods have been described, using various sample types, enrichment media and primer sets (Denis et al., 2001; Englen and Kelley, 2000; Giesendorf and Quint, 1995; Magistrado et al., 2001; Moreno et al., 2001; Ng et al., 1997; O'Sullivan et al., 2000; Waage et al., 1999; Wang et al., 1999; Winters et al., 1997). However, none of them included an internal amplification control (IAC) and none were assessed in collaborative trials, which is the approach of the present study. The lack of proper validation and standardised protocols has hampered the implementation of PCR-based methods by end-user laboratories (Hoorfar and Cook, 2002). In addition, no systematic evaluation is, to our knowledge, available on the interaction of *Campylobacter* enrichment media and pre-PCR treatment on carcass rinse samples, which was the focus of the present study.

The final PCR-based method, including an IAC, was compared with the traditional culture-based method on potentially naturally contaminated chicken-rinse samples. The PCR-based detection method reported here was subsequently validated in a large international multi-centre collaborative trial (Josefsen et al., manuscript submitted).

2. Materials and methods

2.1. Preparation of media

Preston broth (PB) was prepared essentially according to ISO 10272 (Anonymous, 1995). Bolton broth (BB) broth was prepared according to the recommendations of the Bacteriological Analytical Manual Online (Hunt et al., 1998). Mueller–Hinton broth (MHB) was prepared from 21 g of Bacto MHB (Difco, Detroit, USA), 0.25 g of pyruvic acid (Sigma), 0.25 g of sodium bisulphite (Sigma) and 0.25 g of iron sulphate (Sigma) dissolved in 1000 ml of distilled water. The broth was autoclaved at 121 °C for 15 min and the following antibiotics were added aseptically: sodium cefoperazone (30 mg/l, Sigma) and trimethoprim lactate (50 mg/l, Sigma). The blood agar (Oxoid), the modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) and the Preston agar (Oxoid) were prepared according to the manufacturer's instructions.

2.2. DNA extraction

2.2.1. Resin-based extraction

The resin-based extraction was performed essentially as described by Walsh et al. (1991) and modified by Malorny et al. (2003). In brief, 1 ml enriched broth sample (Experiments 2–4) was centrifuged for 5 min at 10,000 × *g* at 4 °C. The pellet was resuspended in 300 μl of 6% Chelex 100 resin suspension (Bio-Rad Laboratories, CA, USA) and incubated for 20 min in a 56 °C water bath. The sample was vortexed for 10 s and incubated in a 100 °C water bath for 8 min, followed by immediate chilling on ice. The sample was centrifuged for 5 min at 14,000 × *g* at 4 °C and 5 μl of the supernatant was used as template in the PCR assay.

2.3. Extraction by boiling

One milliliter enriched sample (Experiment 2) was centrifuged for 5 min at 10,000 × *g* at 4 °C and pellet was resuspended in 1 ml physiological saline. The sample was centrifuged again for 5 min at 10,000 × *g* at 4 °C and the pellet was resuspended in 100 μl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8). The sample was incubated in a 100 °C water bath for 10 min. After a final centrifugation (5 min at 10,000 × *g* at 4 °C), 5 μl of the supernatant was used as template in the PCR assay.

2.3.1. Untreated broth

Five microliters of untreated enriched broth was used as template in the PCR assay.

2.4. PCR conditions

The PCR procedure was based on amplification of a 287-bp sequence of the 16S rRNA gene of *C. jejuni*, *C. coli* and *Campylobacter lari*, and an IAC of 124 bp (Lübeck et al., 2003a,b). PCR amplifications were carried out in 25 μl mixtures, each consisting of 2.5 μl 10 × PCR buffer for T*th* DNA polymerase (Roche Applied Science, Hvidovre, Denmark), 1 U T*th* DNA polymerase (Roche Applied Science), 0.4 mM dNTP nucleotide mixture (Amersham Pharmacia Biotech, Buckinghamshire, UK), 11 pmol forward primer OT 1559 (CTG CTT AAC ACA AGT TGA GTA GG; Lübeck et al., 2003a), 12 pmol reverse primer 18-1 (TTC CTT AGG TAC CGT CAG AA; Lübeck et al.,

2003a), 5 μg bovine serum albumin (BSA; Roche Applied Science) and 50 μmol $MgCl_2$ (Applied Biosystems, Nærum, Denmark). Each reaction tube was added with 5 μl sample containing template DNA. The PCR amplifications were performed in a GeneAmp® PCR Systems 9700 (Perkin Elmer Applied Biosystems, Norwalk, USA) in 0.2-ml thermo-strips (ABgene House, Surrey, UK). Amplification conditions were primary denaturation at 94 °C for 2 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 15 s and extension at 72 °C for 30 s. A final step of 4 min at 72 °C was included to ensure full extension of the products. A negative-control-added 5 μl PCR-grade water was included in each run. A positive PCR result was accepted if a correct-sized band was present. A negative PCR result was accepted only if an IAC band was present.

2.5. Experimental design

Table 1 shows the outline of the experimental design. All incubations were carried out at 42.0 ± 1.0 °C under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2 and 80% N_2), unless stated otherwise.

The number of colony forming units (CFU) in cultures was determined by spreading 100 μl of 10-fold serial dilutions onto blood agar, incubating the agar plates for 24 h and counting them, unless stated otherwise.

2.5.1. Experiment 1: selection of enrichment broth

The growth of different strains of *C. jejuni*, *C. coli* and *C. lari* in PB, MHB and blood-free BB was examined.

The following *Campylobacter* strains were plated onto blood agar and incubated for 24 h: *C. jejuni* strains—Culture Collection University of Göteborg (CCUG) 10935, CCUG 10936, CCUG 10937, CCUG 10938, and 1677; *C. coli* strains—CCUG 10939, CCUG 15360, CCUG 10960, CCUG 11283 and 3931; and *C. lari* strains—CCUG 22395, CCUG 20707, CCUG 23947, CCUG 12774, and 98-40052. From the agar plates, overnight cultures were made in PB, MHB or BB, respectively. PB, MHB or BB were spiked with 100–1000 CFU/10 ml *C. jejuni*, *C. coli* or *C. lari* from the appropriate overnight culture and incubated. The growth was monitored over a period of 24 h, by spreading 10-fold serial dilutions onto blood agar after 0, 4, 10 or 24 h of enrichment.

2.5.2. Experiment 2: spiked samples

The purpose of the experiment was to evaluate the growth and PCR detection of *C. jejuni* 1677 and *C. coli* 3931 in PB, MHB and blood-free BB when chicken-rinse or pork swab-rinse was added. *C. jejuni* 1677 and *C. coli* 3931 were chosen as they are among the most frequently isolated thermotolerant campylobacters from poultry and pork, respectively, in Denmark. At the same time, the ability of the three enrichment media to suppress background flora was investigated. *C. jejuni* 1677 or *C. coli* 3931 were inoculated into 10 ml of PB, MHB or blood-free BB, respectively, and incubated overnight. Two milliliters of chicken-rinse or pork swab-rinse was added to 18 ml of PB, MHB or blood-free BB. The chicken-rinse was prepared as recommended by ISO/CD 6887 (Anonymous, 2000), by washing a whole chicken in 500 ml of cold (4 °C) physiological saline for 60 s

Table 1
Outline of the experimental design for interaction of media and sample treatment for PCR-based detection of thermotolerant campolybacters

Experiment number	Sample type		Enrichment broth				Inoculation strain		
	Chicken-rinse	Pork swab-rinse	PB	MHB	BB including blood	BB not including blood	*C. jejuni*	C. coli	C. lari
1. Selection of enrichment broth			×	×		×	×	×	×
2. Spiked samples	×	×	×	×		×	×	×	
3. Optimisation of enrichment time	×	×			×		×	×	
4. Naturally contaminated samples	×				×				

×: Included in the experiment; PB: Preston broth; MHB: Mueller–Hinton broth; BB: Bolton broth.

(Josefsen et al., 2002). The pork swabs, sampled in accordance with ISO/FDIS 17604 (Anonymous, 2003), by swabbing pork carcass areas of 1400 cm^2 with sterile gauze swabs (10 × 10 cm; Smith & Nephew, Nærum, Denmark), were obtained from the Danish Meat Research Institute. A total of 100 ml of cold (4 °C) physiological saline was added to a stomacher bag containing the pork swab, and it was shaken and rubbed by hand for 60 s. By cutting the corner of the stomacher bag, the pork swab-rinse was transferred to a sterile bottle. The broths were spiked with 0, 1–10, 10–100 and 100–1000 CFU *C. jejuni* 1677 or *C. coli* 3931 from the appropriate overnight culture and incubated. The growth was monitored over 24 h by spreading 10-fold serial dilutions onto mCCDA after 0, 4, 10 or 24 h of enrichment. At the beginning and the end of enrichment, 100 μl were spread onto blood agar to assess the level of background flora. Samples for PCR amplification were drawn after 0, 4, 10 or 24 h of enrichment. PCR was performed on untreated enrichment broth, on DNA extracted by the resin-based method and on DNA extracted by boiling (Fig. 1).

2.5.3. Experiment 3: optimisation of enrichment time

In order to find the shortest time of enrichment necessary for PCR detection, the growth of *C. jejuni* 1677 and *C. coli* 3931 in blood-containing BB, to which chicken-rinse or pork swab-rinse was added, was examined over time. *C. jejuni* 1677 or *C. coli* 3931 was inoculated into 10 ml of blood-containing BB and incubated overnight. To 18 ml of blood-containing BB was added either 2 ml of chicken-rinse or 2 ml of pork swab-rinse, and they were spiked with 0, 1–10, 10–100 and 100–1000/20 ml CFU *C. jejuni* 1677 or *C. coli* 3931 from the overnight culture and incubated. The growth was monitored over a period of 24 h by spreading 10-fold serial dilutions onto mCCDA and blood agar after 0, 12, 18 or 24 h of enrichment. Samples for PCR amplification were drawn at 0 h, and after 12, 14, 16, 18, 20, 22 or 24 h of enrichment. PCR was performed on untreated enrichment broth and on DNA extracted by the resin-based method (Fig. 2).

The effect of an undefined background flora on the growth of *C. jejuni* 1677 and *C. coli* 3931 was investigated. Chicken-rinse was plated on to blood agar and incubated at 37.0 ± 1.0 and 42.0 ± 1.0 °C

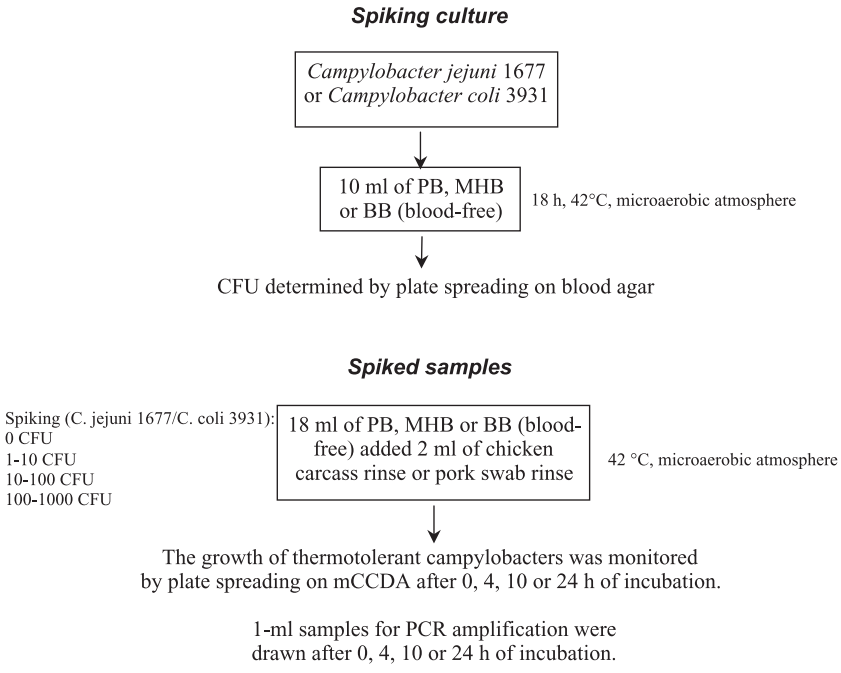

Fig. 1. Flow diagram of the protocol of Experiment 2 on spiked chicken-rinse samples. PB: Preston broth; MHB: Mueller–Hinton broth; BB: Bolton broth.

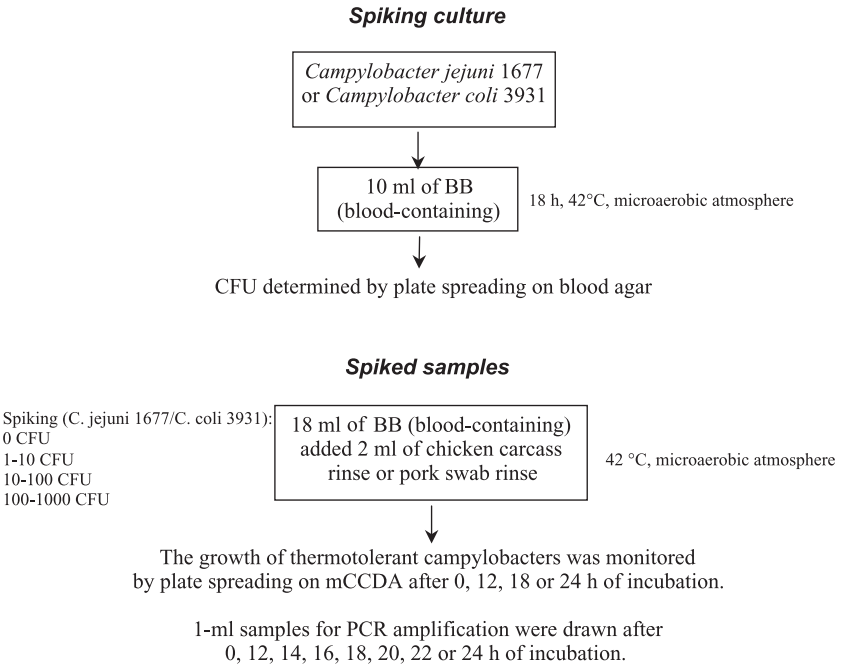

Fig. 2. Flow diagram of the protocol of Experiment 3 for optimisation of enrichment time. PB: Preston broth; MHB: Mueller–Hinton broth; BB: Bolton broth.

under aerobic conditions for 24 h. A suspension of this growth on the agar plates was made in physiological saline and $>10^5$ CFU (total aerobic bacteria) was inoculated into samples spiked with 0 and 100–1000/20 ml CFU *C. jejuni* 1677 and *C. coli* 3931, respectively.

2.5.4. Experiment 4: naturally contaminated samples

In order to compare the performance of the final PCR-based protocol with a standard culture-based method, 66 chickens and 2 ducks were tested, employing both methods. A total of 66 chickens, including 26 Danish and 40 French (imported) free-range or intensively reared chickens and two ducks (Denmark), were purchased on six different occasions from local retailers. The poultry-rinse was prepared as described in Experiment 2, except for the ducks that were rinsed in 1000 ml of saline. Twenty-five milliliters of the poultry-rinse was transferred to 225 ml of blood-containing BB and incubated for 20 h. A 10-fold dilution series was made and 100 μl of this spread on to mCCDA and Preston agar in the concentrations 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. The agar plates were incubated for 48 h. Five typical colonies from each poultry-rinse were verified by applying the following tests: Gram reaction by the KOH 3% method, oxidase test, catalase test and by microscopic inspection of motility and morphology (Josefsen et al., 2002). DNA was extracted by the resin-based method from 1 ml enrichment and examined by PCR.

2.5.5. Data analysis

The PCR-based method was compared to the culture-based method by performing McNemar's test (Siegel and Castellan, 1988). McNemar's test was used to analyse the non-independent paired samples in order to determine the agreement or difference between the two methods.

3. Results

3.1. Selection of enrichment media

All three enrichment media supported the growth of the five *C. jejuni* strains tested equally well.

However, for *C. coli* 3931 (Fig. 3) and *C. coli* CCUG 10939, the growth levels after 24 h were in the range of 10^4–10^5 CFU/ml in PB compared to 10^7–10^8 CFU/ml in both BB and MHB. For *C. coli* CCUG 15360 and *C. coli* CCUG 11283, no growth was detected in PB after 24 h (data not shown). The growth of the five *C. coli* strains tested was supported equally well by BB and MHB. The five *C. lari* strains tested grew in the range of 10^4–10^5 CFU/ml in all three enrichment media, showing no difference among the media tested.

3.2. Spiked samples

C. jejuni 1677 grew equally well in all three media when spiked at higher levels (10–100 and 100–1000 CFU). However, blood-free BB appeared to be superior than PB and MHB in supporting the growth of *C. jejuni* 1677 in the low level (1–10 CFU, data not shown). In the non-spiked samples, the level of growth found on blood agar resembled that on mCCDA, indicating that the majority of colonies were thermotolerant campylobacters.

C. coli 3931 grew equally well in the range of 10^6–10^7 CFU/ml in the three media, regardless of the level of spiking. High levels of non-*Campylobacter* background flora were registered on blood agar from PB and MHB, but not from BB (data not shown).

C. jejuni 1677 was detectable by PCR after 24 h of enrichment regardless of media and sample preparation (Fig. 4). The amplicons obtained from the PB enrichments were weaker than those obtained from BB and MHB. PCR detection in untreated PB was poor. The best PCR detection was obtained when the DNA was extracted from the samples either by the resin-based method or by boiling. The high spiking levels of *C. coli* 3931 were detectable by PCR as early as after 10 h of enrichment. After 24 h of incubation, all levels of spiking were PCR-detectable. Clearer and stronger PCR amplicons were obtained using extracted DNA, especially DNA extracted by the resin-based method. No difference was observed in the PCR results between the three enrichment media.

3.3. Optimisation of enrichment time

No thermotolerant campylobacters were detected by culture in the non-spiked samples, i.e. the samples were culture-negative. For *C. jejuni* 1677, the spiking level had little effect on the level of growth after 24 h of enrichment. For *C. coli* 3931, the level of growth reflected the spiking level after 24 h of enrichment (data not shown). In the non-spiked samples added background flora, no bacterial growth was detected on either blood agar or mCCDA. The samples spiked with 100–1000 CFU, to which background flora were added, reached the same level of growth after 24 h of incubation as the parallel samples without background flora. This was the case for both *C. jejuni* 1677 and *C. coli* 3931. At time zero, the non-spiked samples were negative in the PCR assay. Both *C. jejuni* 1677 and *C. coli* 3931 were detectable by PCR when the growth

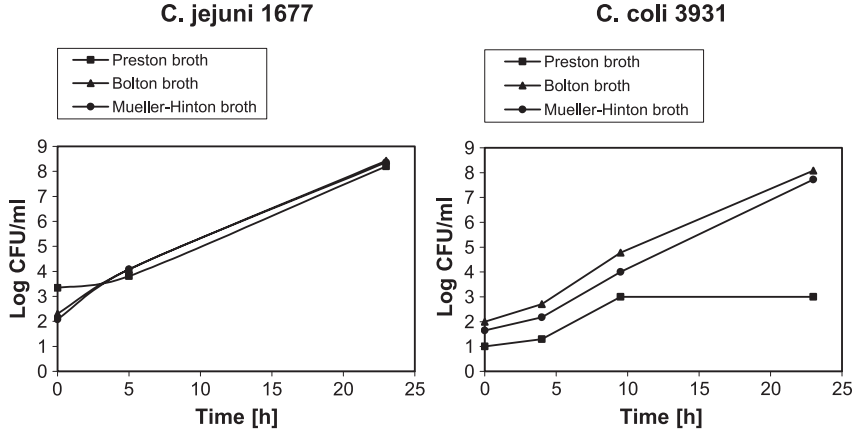

Fig. 3. Typical time-course of growth of *C. jejuni* and *C. coli* in different enrichment broths at 42.0 ± 1.0 °C under microaerobic conditions.

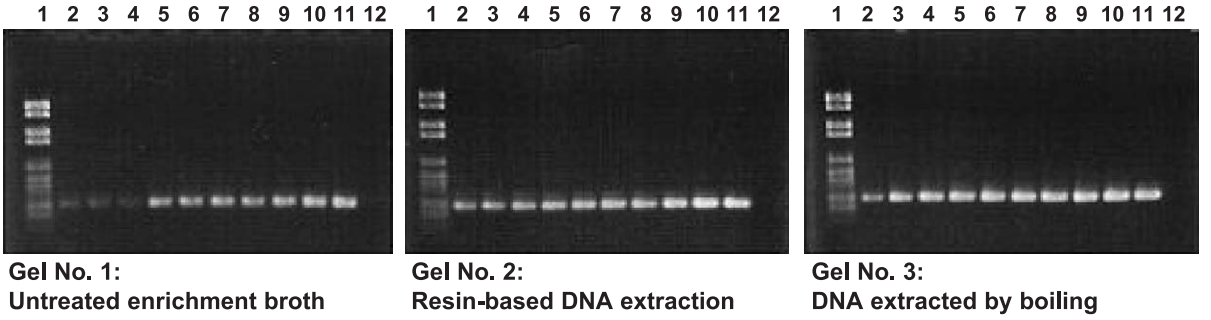

Fig. 4. PCR detection of *C. jejuni* 1677 after 24 h of enrichment. Lane 1: molecular weight marker. Lanes 2–4: Preston broth spiked with 1–10, 10–100 and 100–1000 CFU/20 ml. Lanes 5–7: Mueller–Hinton broth spiked with 1–10, 10–100 and 100–1000 CFU/20 ml. Lanes 8–10: blood-free Bolton broth spiked with 1–10, 10–100 and 100–1000 CFU/20 ml. Lane 11: positive (*C. jejuni* CCUG 11284) DNA control. Lane 12: blank (water) control.

had reached 10^4–10^5 CFU/ml, corresponding to a detection limit of 50–500 CFU per PCR tube. The time window of detection was 12–24 h (testing beyond 24 h was not performed) of enrichment for the samples spiked with 10–100 and 100–1000 CFU and 16–24 h for the samples spiked with 1–10 CFU.

3.4. Naturally contaminated samples

Among the 68 presumably naturally contaminated poultry-rinse samples, 28 were found negative and 40 positive by culture. When tested in the final PCR-based method, 29 samples were negative and 39 positive.

The diagnostic sensitivity of the PCR-based method, defined as the ability to detect thermotolerant *Campylobacter* when detected by the reference method, was 97.5%. The diagnostic specificity, defined as the ability to not detect thermotolerant *Campylobacter* when not detected by the reference method, was 100%.

Comparing the culture-based and the PCR-based methods using McNemar's test, the methods performed equally well ($P=0.32$) in detecting the presence of thermotolerant *Campylobacter* in chicken-rinse and pork swab-rinse.

4. Discussion

In general, the strength of PCR is its ability to rapidly screen for negative samples, thereby giving the possibility to focus on supposedly positive samples. The PCR-based method showed a similar diagnostic accuracy to the standard culture method on naturally contaminated samples. To our knowledge, this is the first international collaborative trial validated, non-commercial PCR-based method, based on the ISO enrichment procedure, making retrospective confirmation of PCR results possible. Furthermore, in compliance with the draft standard on PCR for the detection of foodborne pathogens (ISO/DIS 22174), the method includes a simple sample treatment and an IAC, making it suitable for accreditation in end-user laboratories with quality assurance programs (Anonymous, 2002b).

Preston broth, a common choice of enrichment media in isolating thermotolerant *Campylobacter*, was poor in supporting the growth of *C. coli*. It has also been shown by Ng et al. (1985) that some *C. coli* strains are strongly inhibited by the combination of antibiotics present in PB. Blood-free BB was identified as the most suitable enrichment media, based on its superiority to MHB when low levels of spiking were employed, either with chicken-rinse or pork swab-rinse.

According to the draft standard on PCR testing (ISO/DIS 22174), any enrichment step prior to PCR should follow the standard culture protocol in order to facilitate its implementation in the routine laboratory and provide the possibility of retrospective culture confirmation on the very same samples (Hoorfar and Cook, 2002). As blood-containing BB is currently being accepted as a new enrichment medium in ISO 10272 (Anonymous, 2002a), we evaluated its effect on the growth of thermotolerant *Campylobacter*. Although blood had no detrimental influence on the

growth, it did, as expected, cause some inhibition in the PCR detection (data not shown). However, as this could be overcome by extracting the DNA from the enriched broth samples, blood-containing BB was found to be suitable for enrichment-PCR.

Another finding was that extracting the bacterial DNA by the quick and simple resin-based method reduced inhibition by media or sample material. For enrichment-PCR detection of foodborne thermotolerant *Campylobacter*, several methods have been published (Denis et al., 2001; Giesendorf et al., 1992; Thunberg et al., 2000), although they are labour-intensive. The method presented here will be easier to implement in routine analysis.

Because the level of *Campylobacter* in chicken-rinse is often low, PCR-based detection has to be preceded by enrichment. The only available publication on *C. jejuni* PCR testing of non-enriched chicken rinse deals with highly contaminated chicken samples from the Chinese market containing more than 10^5 campylobacters/ml (Yang et al., 2003). The *Campylobacter* contamination level in chicken products in countries with more hygienic production methods is known to be much lower, making enrichment of most samples necessary for PCR detection. Enrichment ensures a higher level of detection by increasing the number of target cells and diluting the food-derived inhibitory substances (Hoorfar and Cook, 2002). Furthermore, as DNA from non-viable cells will be amplified by PCR as well, enrichment will reduce the chance of detection of dead cells by reducing the ratio between dead and living cells (Mandrell and Wachtel, 1999). On the other hand, several *Campylobacter* enrichment media also contain PCR-inhibitory substances as well, for instance blood (Al-Soud et al., 2000; Al-Soud and Rådström, 2001), bile salts, esculin, acriflavin (Rossen et al., 1992), charcoal and iron (Thunberg et al., 2000). An advantage of introducing the resin-based extraction step in the PCR protocol may be its wider range of application to other food samples and media, which contains different inhibitory substances.

Interestingly, in the present study, the high background flora of chicken or pork samples did not have a negative effect on the growth of *Campylobacter* in blood-containing BB or the PCR response (Experiment 3). The high incubation temperature combined with the selective pressure of the antibiotics present in the media and the microaerobic environment seemed to restrict the growth of the background flora.

In conclusion, a simple, robust and validated PCR-based method for detection of thermotolerant *Campylobacter* in chicken-rinse and pork swab-rinse was developed. A user-friendly sample preparation method was shown to overcome inhibition from the samples and the enrichment media, combined with the use of T*th* DNA polymerase for PCR amplification. T*th* has been proved more resistant to inhibitors than *Taq* DNA polymerase by Laigret et al. (1996) in amplifying *Mycoplasma iowae* from culture medium samples by Poddar et al. (1998) in detecting influenza A virus, by Kim et al. (2001) in amplifying *Staphylococcus aureus* in milk, by Weidbrauk et al. (1995) and by Al-Soud and Rådström (1998). Even low numbers of *Campylobacter* could be detected by PCR after 20 h of enrichment. The method will be proposed as an international standard, as part of the EU-project FOOD-PCR (QLK1-CT-1999-00226).

The PCR-based method presented compares favourably with the ISO culture method and takes two working days to complete, while the ISO culture methods can take up to 5 days to identify negative samples.

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Validation of a PCR-Based Method for Detection of Food-Borne Thermotolerant Campylobacters in a Multicenter Collaborative Trial

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A PCR-based method for rapid detection of food-borne thermotolerant campylobacters was evaluated through a collaborative trial with 12 laboratories testing spiked carcass rinse samples. The method showed an interlaboratory diagnostic sensitivity of 96.7% and a diagnostic specificity of 100% for chicken samples, while these values were 94.2 and 83.3%, respectively, for pig samples.

Meat products are among the primary sources of food-borne campylobacteriosis (24). Rapid detection of *Campylobacter* in these foodstuffs can improve food safety (21). To facilitate implementation of the PCR method by the food industry, it is recommended that the performance characteristics of the tests be thoroughly evaluated through collaborative trials (3).

Thus, a European research project, FOOD-PCR, for validation and standardization of noncommercial PCR-based methods as alternatives to traditional culture-based methods was launched (10).

None of the already published PCR-based methods for detection of thermotolerant *Campylobacter* (6, 7, 18, 19, 22) included an internal amplification control (IAC), nor has any been validated through interlaboratory collaborative trials, which are essential if the method is intended as a diagnostic tool in laboratories with quality assurance programs (3).

In the first stages of the project a sensitive and specific PCR assay for the detection of thermotolerant *Campylobacter* was developed (16) and validated through a multicenter collaborative trial testing purified DNA (17). The assay has been shown to detect all food-borne thermotolerant campylobacters (*Campylobacter jejuni*, *C. coli*, and *C. lari*), which will prepare laboratories for unforeseen shifts in prevalence from one species to the others (16).

To mediate the detection of campylobacters in materials used in primary food production, the PCR assay was incorporated in a complete method, in which the preceding steps were enrichment in Bolton broth followed by a simple and nonproprietary DNA extraction procedure (13). The present paper describes the performance of this method in complex matrices (enriched carcass rinse from chickens and pig swabs), as evaluated in a multicenter trial.

The collaborative trial was designed according to international recommendations (2). Twelve laboratories from the United Kingdom, Austria, Germany, Greece, Slovakia, Denmark, the Czech Republic, Italy, Sweden, The Netherlands, and Norway received 24 coded (blind) 1-ml enriched samples, 12 chicken samples and 12 pig samples, spiked with *C. jejuni* 1677 and *C. coli* 3931, respectively. The samples were spiked at the following levels: 0, 1 to 10, 10 to 100, and 100 to 1,000 CFU/250 ml (Fig. 1). Applying low, medium, and high levels of spiking makes it possible to assess the usefulness of the test at various infection levels. The shipment also included a positive DNA control, an IAC (16), bovine serum albumin (20 mg/ml; Sigma, St. Louis, Mo.), and a resin used for the DNA extraction (Chelex-100; Bio-Rad Laboratories, Hercules, Calif.).

A detailed standard operating procedure (SOP) explaining how to perform the pre-PCR treatment and the PCR assay (see www.pcr.dk for detailed information) was sent to the participating laboratories. The SOP required inclusion of a nontemplate control in every setup. The participants purchased their own primers, DNA polymerase (*Tth*; Applied Biosystems, Foster City, Calif.), and additional reagents from local suppliers. Briefly, the DNA extraction method included short, high-speed centrifugation of 1 ml of enriched broth, addition of Chelex to the pellet, incubation in a 56°C water bath, and final centrifugation (13). The SOP also included a reporting sheet, to be returned to the Central Science Laboratory, Sand Hutton, United Kingdom (not the sending laboratory), for statistical analysis. Participants were required to report in detail all

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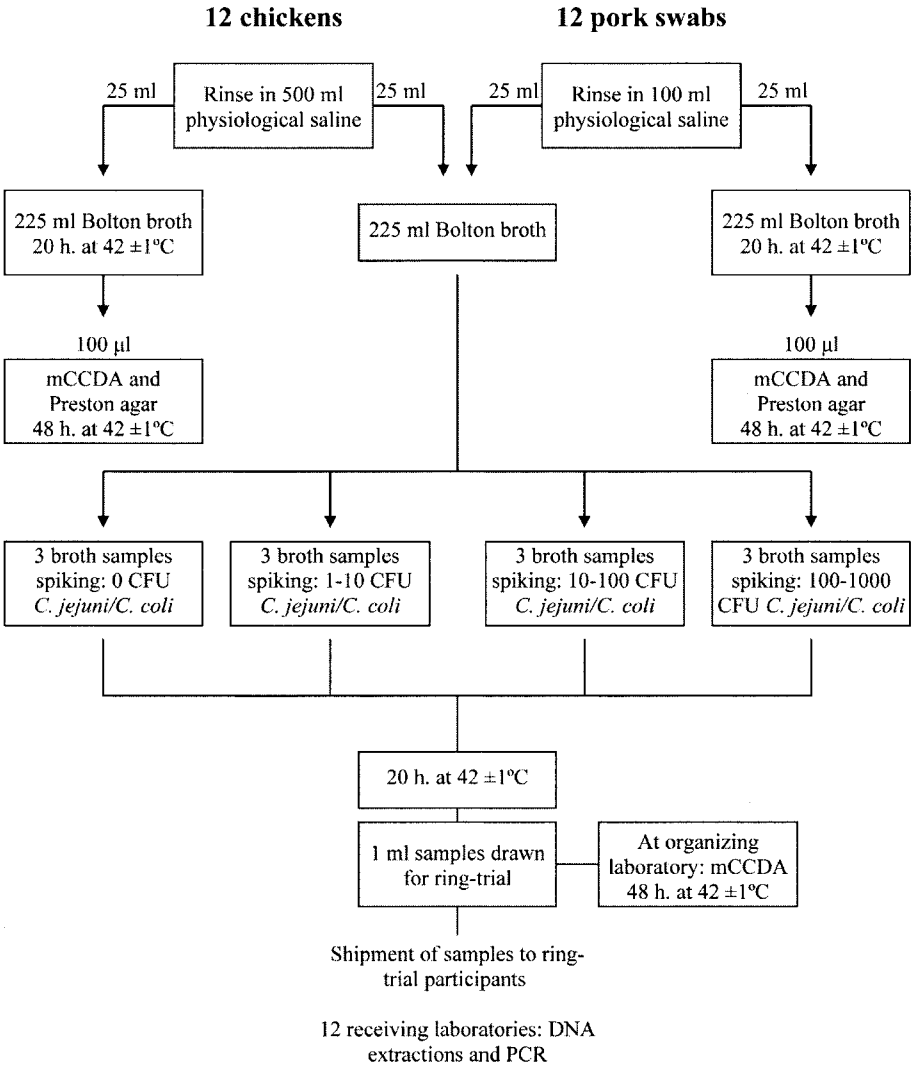

FIG. 1. Flow diagram showing the preparation, spiking, testing, and shipping of samples for the PCR collaborative trial for detection of thermotolerant *Campylobacter*. The chicken samples were inoculated with *C. jejuni*; the pig samples were inoculated with *C. coli*.

additional information that could possibly have influenced their results.

Twelve frozen chickens, declared to be *Campylobacter* free by the producer (Danpo A/S, Aars, Denmark), were purchased at local retailers in Copenhagen, Denmark. Initial suspensions of chicken rinse were prepared as recommended in the ISO/CD 6887-2 protocol (1) and as described by Josefsen et al. (12). The pig carcass swabs, sampled in accordance with ISO/FDIS 17604 (9) by swabbing pig carcass areas of 1,400 cm^2 with sterile gauze swabs, were obtained from the Danish Meat Research Institute (Roskilde, Denmark). Initial suspensions of 12 carcass swabs were prepared by washing the swabs in 100 ml of physiological saline for 60 s (13). As shown in Fig. 1, a total of 25 ml of chicken or pig swab rinse was transferred to 225 ml of blood-containing Bolton broth prepared according to the recommendations of the *Bacteriological Analytical Manual Online* (11). To verify that the chicken and pig swab rinses were initially culture negative, samples were drawn for intensive culturing before the inoculations. This analysis was conducted in accordance with recommendations of both ISO 10272-1 (4) and the Nordic Committee on Food Analysis (20), in order to verify the *Campylobacter*-free status of the samples.

C. jejuni 1677 and *C. coli* 3931 (which are frequently isolated from chickens and pigs, respectively) were inoculated into chicken and pig broth samples, respectively. Three broth samples were left noninoculated, three were inoculated with approximately 1 to 10 CFU/250 ml, three were inoculated with approximately 10 to 100 CFU/250 ml, and three were inoculated with approximately 100 to 1,000 CFU/250 ml. All samples were enriched for 20 h at 42 ± 1°C under microaerobic conditions. One-milliliter broth samples were drawn and stored at −80°C (maximum storage period: 3 weeks), until shipped on dry ice by courier to the participants. Prior to the shipment, identical samples were tested by PCR in the organizing laboratory (Danish Institute for Food and Veterinary Research, Copenhagen, Denmark) according to the SOP to verify the detection of thermotolerant *Campylobacter*.

The participating laboratories mailed the results directly to the Central Science Laboratory (a different laboratory than the organizing laboratory). The gel pictures were carefully exam-

TABLE 1. Reported participant results for the PCR-based method for detection of thermotolerant campylobacters in spiked chicken rinse and pig swab rinse samples

Inoculation level	No. of samples positive for the target PCR amplicon																							
	Expected[a]	From chickens for participant:												From pigs for participant:										
		1	2[b]	3	4	5	6	7[c]	8	9	10	11	12	1	2[b]	3	4	5	6	7[c]	8	9	10	11	12
Noninoculated	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	3	1	0	1	2	0
Low (1–10 CFU)	3	3	0	3	3	3	3	3	3	1	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3
Medium (10–100 CFU)	3	3	0	3	3	3	3	3	3	2	3	3	3	3	0	3	3	3	3	3	3	3	3	2	3
High (100–1,000 CFU)	3	3	0	3	3	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	2	3

[a] From analysis of triplicate samples.
[b] Excluded due to omission of the IAC in the PCR mixture.
[c] Excluded due to the presence of target amplicons in the assay negative control.

ined, and the results were approved for inclusion in statistical analysis unless they fell into one of the following two categories: (i) obvious performance deviation from the SOP and (ii) presence of target amplicons in negative PCR controls, indicating contamination.

Table 1 shows the participants' results for the collaborative trial. In agreement with the predefined criteria, the results of partner 2 were excluded, as this partner did not include the IAC in the PCR mixture and thus was not able to determine whether the samples were truly negative or whether the absence of target amplicons was due to PCR inhibition. The results of partner 7 were also excluded, as partner 7 reported target amplicons in the assay negative control, indicating possible cross-contamination. All remaining results were accepted according to the predefined criteria; thus the final statistical analysis was based on 10 sets of results (Table 2).

The PCR results were analyzed statistically according to the recommendations of Scotter et al. (23) by the method of Langton et al. (14). The interlaboratory diagnostic sensitivity was defined as the percentage of positive samples giving a correct positive signal (8). The interlaboratory diagnostic specificity was defined as the percentage of negative samples giving a correct negative signal, as well as a signal from the IAC (8). Confidence intervals (CI) for interlaboratory diagnostic sensitivity and specificity were calculated by the method of Wilson (25). Accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) were defined as the percentages of finding the same result, positive or negative, from two similar samples analyzed in, respectively, the same or different laboratories under standard repeatability conditions. These calculations take into account different replications in different laboratories by weighting results appropriately. The concordance odds ratio (COR) was defined as the degree of between-laboratory variation in the results. The COR was expressed as the ratio between accordance and concordance percentages. CI for accordance, concordance, and COR were calculated by the method of Davidson and Hinckley (5).

For chicken samples, the interlaboratory diagnostic sensitivity of the PCR was greater than 90% for each of the inoculation levels, and calculating the interlaboratory diagnostic sensitivity for all inoculation levels gave a value of 96.7% (Table 2). Accordance (repeatability) and concordance (reproducibility) values were thus high in each case. With samples inoculated with 1 to 10 CFU, a degree of interlaboratory variation was noted; this was due to one laboratory reporting two false-negative results. The COR value can be interpreted as the likelihood of obtaining the same result from two identical samples, whether they are sent to the same laboratory or to two different laboratories. The closer the value is to 1.0, the higher the likelihood is of obtaining the same result. In all cases, the COR value fell within the 95% CI, indicating that the interlaboratory diagnostic sensitivity of the method was as repeatable as it was reproducible (Table 2). Thus, the method is effective at detecting thermotolerant *Campylobacter* in the sample types tested. Conversely, and more importantly, there is a very low risk of obtaining false-negative responses: the interlaboratory diagnostic specificity, or percentage of correctly identified noninoculated chicken samples, was 100%, with complete accordance and concordance. The PCR-based method reported performed favorably compared to the International Standard Organisation culture method (13). The latter takes 5 days to identify negative samples, while the method reported here takes only two working days. Here it must be emphasized that the main advantage of PCR over culture methods is its potential for rapid screening of negative samples, which allows for greater resources to be directed toward characterization and epidemiological tracking of positive isolates. In end use laboratories using PCR for screening purposes, a confirmation of PCR-positive responses by culture may not be necessary.

A similar approach has been used in Denmark for identification of infected flocks prior to and after slaughter, in order to provide the consumers with campylobacter-free chickens, declared as such. The implementation of the so-called "strategic slaughter," where infected flocks are slaughtered at the end of the day, seems to have contributed to the recent significant decline of human campylobacteriosis in Denmark (www.dzc.dk).

For the pig samples, the interlaboratory diagnostic sensitivity of the PCR was greater than 90% for each inoculation level and calculating the interlaboratory diagnostic sensitivity for all inoculation levels resulted in a value of 94.2% (Table 2). In all cases accordance and concordance values were high, and the CORs showed that the interlaboratory diagnostic sensitivity was equally repeatable and reproducible. For these samples, the interlaboratory diagnostic specificity was 83.3%, reflecting a greater degree of interlaboratory variation in results for pig samples. This was due to four laboratories reporting positive amplicons from noninoculated samples. Since the pig samples had previously been identified as *Campylobacter* free, both by culture and PCR in the sending laboratory, cross-contamination in the receiving laboratories could explain these results. With hindsight, this could have been controlled within the trial

TABLE 2. Statistical evaluation of PCR results obtained for the detection of thermotolerant campylobacters in chicken rinse and pig swab rinse samples[a]

Inoculation level	Sensitivity (%)		Specificity (%)		Accordance (%)		Concordance (%)		COR	
	Pig	Chicken	Pig	Chicken	Pig	Chicken	Pig	Chicken	Pig	Chicken
Noninoculated	—[b]	—	83.3 (66.4, 92.7)	100 (88.7, 100)	73.3 (53.3, 93.3)	100	71.1 (55.8, 93.3)	100	1.12 (0.73, 1.67)	1.00
Low	100 (88.7, 100)	93.3(78.7, 98.2)	—	—	100	93.3 (80.0, 100)	100	86.7 (65.9, 100)	1.00	2.15 (1.00, 2.15)
Medium	96.7 (83.3, 99.4)	96.7 (83.3, 99.4)	—	—	93.3 (80.0, 100)	93.3 (80.0, 100)	93.3 (81.5, 100)	93.3 (81.5, 100)	1.00 (0.91, 1.00)	1.00
High	96.7 (83.3, 99.4)	100 (88.7, 100)	—	—	93.3 (80.0, 100)	100	93.3 (81.5, 100)	100	1.00 (0.91, 1.00)	1.00
All positive levels	94.2 (88.5, 97.2)	96.7 (90.7, 98.9)	—	—	96.1 (88.3, 100)	95.0 (85.0, 100)	95.6 (87.3, 100)	93.3 (81.5, 100)	1.15 (1.00, 1.15)	1.36 (1.00, 1.36)

[a] Numbers in parentheses are the lower and upper 95% CI values, respectively.
[b] —, not applicable.

by including a processing negative control (PNC), e.g., a sample comprising sterile water, which underwent treatment identical to that of the samples. A positive result from a PNC sample would reveal the occurrence of cross-contamination. This is a drawback of the present study, which should be avoided in similar trials in the future. In addition, if the method is to be performed routinely, it is strongly recommended that such controls be included throughout the entire test process, including sample preparation, enrichment, DNA extraction, and target amplification (3). However, in routine application of the PCR method, any positive response should be confirmed by reanalyzing the retained Bolton broth culture by the ISO method.

To our best knowledge, no other collaborative trial has validated a similar noncommercial, open-formula PCR for *Campylobacter* (15). The method does not require procurement of costly equipment. These features, in combination with the validation presented here, make it suitable for routine use and thus appropriate for accreditation.

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Enrichment Followed by Quantitative PCR both for Rapid Detection and as a Tool for Quantitative Risk Assessment of Food-Borne Thermotolerant Campylobacters

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As part of a large international project for standardization of PCR (Food-PCR; www.pcr.dk), a multiplex, multiplatform, ready-to-go enrichment followed by a real-time PCR method, including an internal amplification control, was developed for detection of food-borne thermotolerant campylobacters in chickens. Chicken rinse samples were enriched in Bolton broth for 20 h, a simple and rapid (1-h) resin-based DNA extraction was performed, and DNA samples were then tested with two instrument platforms: ABI-PRISM 7700 and RotorGene 3000. The method was validated against an International Standard Organization (ISO)-based culture method by testing low, medium, and high levels of 12 spiked and 66 unspiked, presumably naturally contaminated, chicken rinse samples. In the RotorGene, a positive PCR response was detected in 40 samples of the 66. This was in complete agreement with the enriched ISO culture. The ABI-PRISM 7700 missed one culture-positive sample. Positive samples contained 10^2 to 10^7 CFU/ml after enrichment in Bolton broth. In the enriched samples a detection probability of 95% was obtained at levels of 1×10^3 and 2×10^3 CFU/ml in the RotorGene and ABI-PRISM, respectively. The amplification efficiency in both platforms was 90%, although the linear range of amplification of purified genomic DNA was 1.5×10^1 to 1×10^7 ($R^2 = 1.00$) for the RotorGene and 10^3 to 10^7 ($R^2 = 0.99$) for the ABI-PRISM. In RotorGene and ABI-PRISM the levels of precision of detection as determined by standard deviation (coefficients of variation) of 6-carboxyfluorescein (FAM) threshold cycle (Ct) values were 0.184 to 0.417 (0.65 to 2.57%) and 0.119 to 0.421 (0.59 to 1.82%), respectively. The results showed a correlation (R^2) of 0.94 between the target FAM Ct values and CFU per milliliter of enriched naturally contaminated chicken samples, which indicates PCR's additional potential as a tool for quantitative risk assessment. Signal from the internal amplification control was detected in all culture-negative samples (VIC Ct: 23.1 to 28.1). The method will be taken further and validated in an international collaborative trial with regard to standardization.

Thermotolerant campylobacters especially *Campylobacter jejuni* and *C. coli* are recognized worldwide as a leading cause of human food-borne infections (19). They are zoonotic bacteria, with many wild and domesticated animals serving as potential reservoirs. Sources of sporadic campylobacteriosis are seldom identified, but water, pets, and especially poultry products are known to be associated with the illness (14). There is a need for rapid detection methods during chicken production, in order to prevent distribution of infected chicken products to consumers. Rapid methods, as currently practiced in Denmark, can be used on-site to quickly identify infected flocks prior to and after slaughter and to provide the consumers with *Campylobacter*-free chickens. The implementation of the so-called "strategic slaughter" seems to have contributed to the recent decline of campylobacteriosis in Denmark (www.dfvf.dk). In addition, quantitative detection tools are needed for estimation of the level of *Campylobacter* in flocks and meat products, as part of risk assessment studies.

A limited number of real-time PCR methods have been reported for the specific detection of *C. jejuni* (5, 15, 18, 20). The present study deals with detection of all food-borne thermotolerant campylobacters (*C. jejuni*, *C. coli*, and *C. lari*), which would prepare laboratories for an unforeseen shift of prevalence in poultry from *C. jejuni* to currently less frequently isolated species. In addition we have included an internal amplification control (IAC), which is mandatory according to the draft international standard (4). Furthermore, the real-time PCR method reported here is based on a specific and robust conventional gel-based PCR method validated in an international collaborative trial (10, 11). Closed-tube real-time PCR assays can be more specific than gel-based PCR due to the inclusion of a target sequence-recognizing probe. They can also be faster and more sensitive and involve less manual handling than conventional PCR. Finally, the risk of carryover contamination is substantially reduced, which makes them more suitable for implementation in end use laboratories.

To our knowledge, this is the first study to report an enrichment multiplatform real-time PCR, including an IAC, which deals with the detection of all three food-borne thermotolerant campylobacters and to provide data to support its potential use as a quantitative tool in risk assessment studies.

MATERIALS AND METHODS

Spiked and naturally contaminated samples. (i) Spiked samples. The PCR method was validated on 12 frozen slaughtered chickens (Danpo A/S, Aars, Denmark), purchased at local retailers as *Campylobacter* free. Initial suspensions of chicken carcass rinse were prepared as recommended in the ISO/CD 6887-2 protocol (1). A whole thawed chicken was placed in a large sterile plastic bag, 500

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ml of physiological saline (0.9% NaCl) was added, and the bag was closed and shaken gently by hand for a minimum of 60 s (8). Then 25 ml of chicken carcass rinse was transferred to 225 ml of Bolton broth (BB) prepared according to the recommendations of the *Bacteriological Analytical Manual Online* (6). The broth samples were inoculated with *C. jejuni* strain 1677, which represents a genotype frequently isolated from chickens in Denmark. Three broth samples were not inoculated; three were inoculated with approximately 1 to 10 CFU/250 ml, three were inoculated with approximately 10 to 100 CFU/250 ml, and three were inoculated with approximately 100 to 1,000 CFU/250 ml. They were incubated at 42.0 ± 1.0°C under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, and 80% N_2) for 20 h. Following the enrichment, the concentrations of *C. jejuni* 1677 in the spiked samples were approximately 0, 10^4, 10^5, and 10^6 CFU/ml. After 20 h of enrichment, 1-ml samples were drawn for PCR and stored at −80°C. To verify that the chicken carcass rinse was initially *Campylobacter* free, prespiking samples were drawn for traditional microbiological analysis before inoculation. The microbiological analysis was conducted in accordance with recommendations of the International Standard Organization (3) and the Nordic Committee on Food Analysis (2). The CFU levels postenrichment were determined by plating 100 μl of a 10-fold dilution series (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) onto modified charcoal cefoperazone deoxycholate agar (Oxoid, Basingstoke, United Kingdom) and Preston agar (Oxoid). The agar plates were incubated for 48 h at 42.0 ± 1.0°C under microaerobic conditions. Five typical colonies from each plate were verified by applying the following tests: Gram reaction by the KOH 3% method, oxidase test, catalase test, and microscopic inspection of motility and morphology (8).

(ii) Naturally contaminated samples. Sixty-six chickens, including 26 Danish and 40 French free-range or intensively reared chickens, were purchased on six different occasions at local retailers. The chicken carcass rinses were prepared as described above. Twenty-five milliliters of the chicken carcass rinse was transferred to 225 ml of BB and enriched for 20 h, after which 1-ml samples were drawn for PCR and stored at −80°C. Microbiological analysis was conducted as described in the section for spiked samples.

Preparation of template DNA. A simple and rapid resin-based sample treatment was performed essentially as described by Malorny et al. (13). Briefly, the 1-ml frozen enriched BB samples were thawed and centrifuged for 5 min at 10,000 × *g* and 4°C. The pellets were resuspended in 300 μl of 6% Chelex 100 resin suspension (Bio-Rad Laboratories, Hercules, Calif.) and incubated for 20 min in a 56°C water bath. The samples were vortexed for 10 s and incubated in a 95°C water bath for 8 min, followed by immediate chilling on ice. The samples were centrifuged for 5 min at 14,000 × *g* and 4°C, and 5 μl of the supernatants was used as the template in the PCR. The spiked and naturally contaminated samples were run in duplicate in an ABI-PRISM 7700 sequence detector (Applied Biosystems, Foster City, Calif.) and a RotorGene 3000 (Corbett Research, Mortlake, Australia). Preparation of the IAC template (hemorrhagic septicemia virus mRNA for envelope protein; GenBank accession no. X66134) was performed as previously described (10).

***Campylobacter* real-time PCR.** The real-time PCR was performed in the ABI-PRISM using 0.2-ml thermostrips (ABgene House, Surrey, United Kingdom) or MicroAmp Optical 96-well reaction plates (Applied Biosystems) closed with MicroAmp Optical caps (Applied Biosystems) and in the RotorGene using 0.2-ml capped tubes (Corbett Research). The 25-μl real-time PCR mixture contained 1× PCR buffer for *Tth* DNA polymerase (Roche A/S, Hvidovre, Denmark), 1 U of *Tth* DNA polymerase (Roche A/S), 0.4 mM deoxynucleoside triphosphate mixture (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), 0.44 μM forward primer 5′ CTG CTT AAC ACA AGT TGA GTA GG 3′, 0.48 μM reverse primer 5′ TTC CTT AGG TAC CGT CAG AA 3′ (DNA Technology, Århus, Denmark; *C. jejuni* 16S rRNA; GenBank accession no. Y19244), 2.5 mM $MgCl_2$ (Applied Biosystems), 30 μg of bovine serum albumin (BSA) for chicken samples and 5 μg of BSA for pure DNA (Roche A/S), 20 nM target *Campylobacter* probe labeled with 6-carboxyfluorescein (FAM; reporter dye) and 6-carboxytetramethylrhodamine (TAMRA; quencher dye) (5′ FAM-TGT CAT CCT CCA CGC GGC GTT GCT GC-TAMRA 3′; DNA Technology), 50 nM IAC probe (5′ VIC-TTC ATG AGG ACA CCT GAG TTG A-TAMRA 3′; Applied Biosystems), 5 × 10^3 copies of IAC (124 bp), and 5 μl of DNA sample. The cycle profile was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 58°C for 60 s. Fluorescence measurements were obtained online and analyzed on the ABI-PRISM with the SDS software (version 1.7a; Applied Biosystems) and on the RotorGene with the version 4.6 software (Corbett Research). Note that no passive reference fluorescence was assigned on the ABI-PRISM, since the PCR mixture did not contain any 6-carboxy-X-rhodamine. The real-time PCR method is based on a specific and robust conventional gel-based PCR method validated in an international collaborative trial. No attempt was made to analyze the specificity of the test since the primers used previously have been validated against 150 related and nonrelated species (10, 11).

Determination of cutoff. The FAM threshold was assigned to a fixed value intersecting the amplification curves in the linear region of the logarithmic plot (a normalized reporter signal, ΔRn, of 230 for the ABI-PRISM and a normalized fluorescence of 0.02 for the RotorGene). The fluorescence was normalized to the background signal, as no 6-carboxy-X-rhodamine was present in the master mixture. Any sample showing a fluorescence signal above this value was regarded as positive. The VIC threshold was assigned a normalized reporter signal (ΔRn) of 100 for the ABI-PRISM and a normalized fluorescence of 0.01 for the Rotor Gene. To exclude possible weak signals from samples or nontemplate controls with a slight end point drift upward, a 10% quantitation setting on the RotorGene was employed, meaning that the software would reject any reaction not increasing more than 10% in fluorescence relative to the samples producing the highest fluorescence increase in the real-time run.

Detection probability. The probability of detection was examined essentially as described by Knutsson et al. (9). Six chicken carcass rinse samples containing approximately 10^1 to 10^6 *Campylobacter* CFU/ml were tested. Five microliters of extracted DNA from each sample was added to five separate real-time PCR tubes and was run as described above on both the ABI-PRISM and the RotorGene. This experiment was repeated five times in the same PCR run on six separate occasions, resulting in a total of 30 responses for each chicken sample. Master mixture was prepared on each of the six occasions and divided among five PCR tubes. DNA was added to the tubes individually. The real-time PCR response was regarded as binary, giving either a positive or a negative signal, according to the cutoff criteria described earlier. The detection probability in spiked chicken carcass rinse samples was obtained by plotting the relative percentage of positive PCR responses against the cell concentration in the samples tested. A sigmoid line fitting was performed with ORIGIN, version 4.0 (Microcal Software, Northampton, Mass.).

Precision of detection. Chicken carcass rinse samples spiked with 0, 1 to 10, 10 to 100, and 100 to 1,000 CFU/250 ml, in the presence of 5 × 10^3 copies of the IAC, were used to establish the precision of detection. Five microliters of DNA extracted from three spiked samples from each level was added to 10 separate real-time PCR tubes, and samples were run as described above on both the ABI-PRISM and the RotorGene. For statistical analysis the results for the precision of detection were plotted into the Online Calculator for Standard Deviation (www.compute.uwlax.edu/stats/), and the standard deviation (SD), sample variance (s^2), and the coefficient of variation (CV) were computed.

Amplification efficiency and linear range. The amplification efficiency and linear range of the real-time PCR method were determined in quadruple by using purified DNA from plate colonies in the concentration range of 5 × 10^0 to 1 × 10^7 copies of genomic DNA from type strain *C. jejuni* CCUG 11284. The analysis was performed on both the ABI-PRISM and the RotorGene. By plotting the number of genomic DNA copies/25 μl of PCR sample against the threshold cycle value (Ct; the fractional PCR cycle at which the fluorescence signal of a sample rises above the determined baseline signal), a linear relationship was formed. The slope of this curve was used to determine the amplification efficiency from the equation amplification efficiency = $10^{-1/\text{slope}} - 1$. The DNA was extracted with a DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined by PicoGreen quantitation of double-stranded DNA with a TD-360 minifluorometer (Turner Designs, Sunnyvale, Calif.) (7). The number of genomic copies of the purified DNA was calculated from the equation $m = n(1.013 \times 10^{-21})$, where m is the mass, and n is the number of base pairs in the genome (12). The genomic sequence of *C. jejuni* NCTC11168 has been determined by Parkhill et al. (16) to be 1,641,481 bp. Entering this in the above equation yields the result that one *C. jejuni* genome weighs approximately 1.7 fg. The number of genomic DNA copies/milliliter was adjusted with 1× Tris-EDTA buffer–0.1 M EDTA.

RESULTS

Amplification efficiency and linear range. The standard curves produced directly by software for both platforms are shown in Fig. 1. The linear range of amplification for the PCR method was between 5 × 10^1 and 1 × 10^7 copies of purified genomic DNA from *C. jejuni* CCUG 11284 in the RotorGene and 10^3 to 10^7 copies in the ABI-PRISM (Fig. 2). The amplification efficiency was computed from the slope of the linear relationship ($R^2 = 1.00$) and was 90.6% for the RotorGene. In

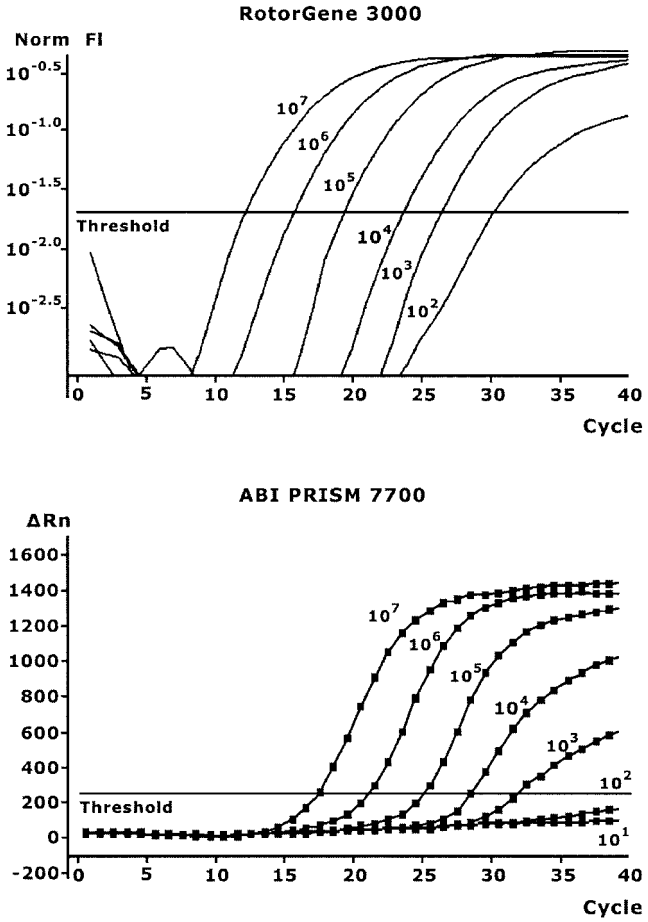

FIG. 1. Standard curves produced from purified DNA in the range of 10^1 to 10^7 copies of genomic DNA from type strain *C. jejuni* CCUG 11284 on the ABI-PRISM 7700 and the RotorGene 3000. The lowest level, 10^1 copies, is not shown for the RotorGene since it was eliminated by the quantification settings of 10%. Norm fl, normalized fluorescence.

the ABI-PRISM the results were 89.9% for amplification efficiency and a linear relationship of $R^2 = 0.99$.

Detection probability. Figure 3 shows the probability of detecting *C. jejuni* 1677 in chicken carcass rinse samples by the real-time PCR method. On the ABI-PRISM the detection probability for 10^3 CFU/ml was 85% and for 4×10^3 CFU/ml it was 100%. On the RotorGene the probability of detecting 10^3 CFU/ml was 90%, while it was 100% for detecting 4×10^3 CFU/ml. The levels of detection at 95% probability were determined to be 2×10^3 CFU/ml for the ABI-PRISM and 10^3 CFU/ml for the RotorGene.

Precision of detection in artificially contaminated chicken rinse samples. To evaluate the precision of detection with the two instrument platforms, a range of identical chicken carcass rinse samples were run simultaneously in the same real-time PCR. As shown in Table 1, the SD, s^2, and CV were low for both the ABI-PRISM and the RotorGene, reflecting a high degree of precision of detection. For the ABI-PRISM the SD and CV ranged from 0.119 to 0.421 and 0.59 to 1.82%, respectively, and for the RotorGene the SD and CV ranged from 0.184 to 0.417 and 0.65 to 2.57%, respectively.

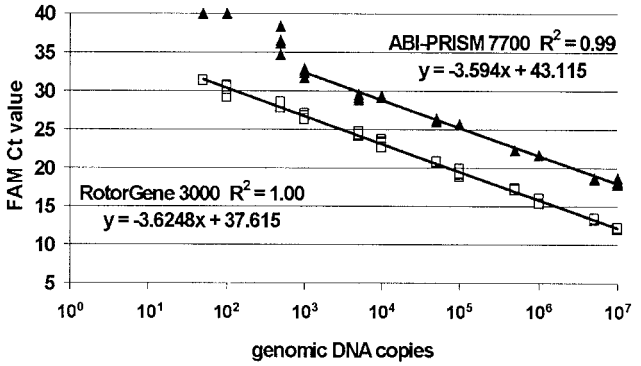

FIG. 2. The linear range of the real-time PCR method when detecting purified DNA from *C. jejuni* CCUG 11284 on the RotorGene 3000 (5×10^1 to 1×10^7 copies; □) and the ABI-PRISM 7700 (10^3 to 10^7 copies; ▲). A real-time PCR sample containing below 5×10^1 genomic DNA copies gave either no positive response or was removed by the detection software because of the 10% quantification correction on the RotorGene. Levels above 10^7 genomic DNA copies were not tested.

Spiked and naturally contaminated samples. The samples spiked with 0, 1 to 10, 10 to 100, and 100 to 1,000 CFU/250 ml reached levels of approximately 0, 10^4, 10^5, and 10^6 CFU/ml, respectively, after 20 h of enrichment. PCR detection was possible on both instruments at all spiking levels. However, in general the RotorGene gave lower FAM Ct values than the ABI-PRISM. Based on all spiking levels, the average FAM Ct value was 6 U lower on RotorGene (Table 2).

Thermotolerant campylobacters were found in 40 out of the 66 presumably naturally contaminated chicken rinse samples by the culture-based method. The same 40 culture-positive samples were also found positive with the RotorGene, whereas the ABI-PRISM gave 39 positive responses. The only sample not detected by the ABI-PRISM contained 1.5×10^2 CFU of thermotolerant campylobacters/ml. There was 100% agreement between the duplicate responses from the 66 naturally contaminated samples. Figure 4 illustrates the linear correla-

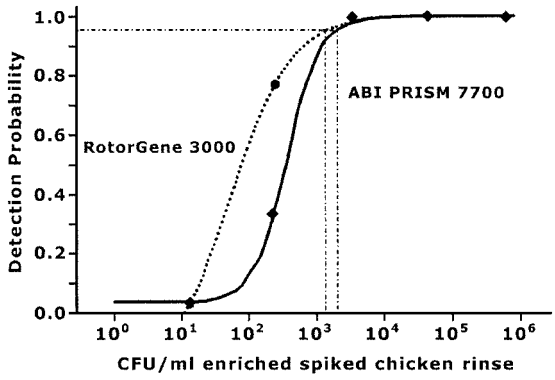

FIG. 3. Detection probability on ABI-PRISM 7700 and RotorGene 3000 for thermotolerant campylobacters with spiked chicken rinse samples containing 10^1 to 10^6 CFU/ml. Thirty responses for each sample were generated on six separate occasions. Thick dotted line, RotorGene 3000; solid line, ABI-PRISM 7700; intersecting thin dotted lines, 95% detection probability. The detection probability was determined in the presence of 5×10^3 copies of the IAC.

TABLE 1. Results from the determination of the precision of detection of *C. jejuni* 1677 in spiked chicken rinse samples[a]

Spiking level (CFU/250 ml)	ABI-PRISM 7700				RotorGene 3000			
	Mean Ct value	SD	s^2	CV (%)	Mean Ct value	SD	s^2	CV (%)
0	40	0	0	0	40	0	0	0
0	40	0	0	0	40	0	0	0
0	40	0	0	0	40	0	0	0
1–10	27.89	0.290	0.084	1.04	22.61	0.365	0.133	1.61
1–10	26.42	0.388	0.151	1.60	20.64	0.293	0.086	1.42
1–10	27.33	0.421	0.178	1.54	21.09	0.283	0.080	1.34
10–100	21.50	0.148	0.022	0.69	14.85	0.184	0.034	1.24
10–100	21.94	0.223	0.050	1.02	16.29	0.417	0.175	2.57
10–100	22.61	0.261	0.068	1.15	16.51	0.371	0.138	2.25
100–1,000	19.45	0.219	0.048	1.12	13.34	0.328	0.108	2.46
100–1,000	20.24	0.119	0.014	0.59	13.66	0.286	0.082	2.09
100–1,000	19.64	0.358	0.128	1.82	13.46	0.296	0.088	0.65

[a] Samples contained 0, 10^4, 10^5, and 10^6 CFU/ml after enrichment.

tion between the Ct values for the target probe and the number of CFU in naturally contaminated chicken samples enriched in BB in both platforms (data available at www.pcr.dk/fig.4.doc). Comparison of real-time PCR with the culture-based method, as applied to the 66 naturally contaminated samples, indicated no difference between the two methods, independent of the real-time platform used. The 26 culture *Campylobacter*-negative samples all showed a FAM Ct value of 40, while the IAC (VIC) signal was detected in all negative samples. VIC Ct values ranged from 26.9 to 28.1 for the ABI-PRISM and 23.1 to 27.9 for the RotorGene.

Analytical accuracy. No attempt was made to analyze the specificity of the tests, since the primers used previously have been validated against 150 related and nonrelated species (11).

TABLE 2. Results of real-time PCR for detection of food-borne thermotolerant campylobacters on spiked chicken rinse samples tested in duplicate on ABI-PRISM 7700 and RotorGene 3000[a]

Level of spiking (CFU/250 ml)	Ct value for:							
	ABI-PRISM 7700				RotorGene 3000			
	Duplicate 1		Duplicate 2		Duplicate 1		Duplicate 2	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
0	40	26.5	40	27.1	40	27.2	40	26.9
0	40	27.0	40	27.0	40	28.4	40	26.6
0	40	26.9	40	26.7	40	28.3	40	26.1
1–10	27.5	27.2	27.3	26.8	21.7	26.9	22.2	26.2
1–10	26.6	29.3	25.4	26.9	20.1	26.1	18.9	26.7
1–10	26.7	27.8	25.3	26.6	19.8	26.6	20.7	25.4
10–100	21.3	29.4	21.1	26.8	15.2	26.3	15.0	25.1
10–100	22.2	28.9	21.2	27.6	14.7	26.0	15.5	25.0
10–100	22.5	28.1	21.2	26.8	16.0	26.1	15.1	25.6
100–1,000	20.7	31.7	19.0	28.1	12.6	27.0	12.6	25.7
100–1,000	19.5	29.7	18.6	28.4	13.0	26.3	13.1	25.5
100–1,000	20.0	27.7	19.0	28.5	13.3	26.8	12.7	28.3

[a] After enrichment the spiked samples contained approximately 0, 10^4, 10^5, and 10^6 CFU/ml.

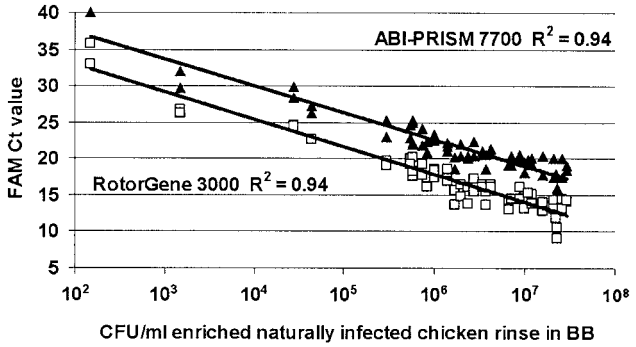

FIG. 4. Linear correlation between target Ct values and CFU in naturally contaminated chicken samples enriched in BB. Samples were tested in duplicate on ABI-PRISM 7700 (▲) and RotorGene 3000 (□).

A complete "ready-to-go mixture" was stored at −20°C up to 1 month and tested on several occasions against freshly prepared master mixture. DNA material from spiked chicken rinse samples was added to the master mixtures, and the resulting FAM Ct values showed no difference throughout the month (data not shown). Furthermore a protection against carryover contamination was achieved by exchanging 2.5 mM dTTP in the nucleotide mixture with 5 mM dUTP (12.5 mM nucleotide mixture including dUTP; Applied Biosystems) and 1/4 U of heat-labile uracil-DNA glycosylase (Invitrogen A/S, Tåstrup, Denmark). For genomic DNA this resulted in FAM Ct values that were between 1 and 3 cycles lower (stronger signal). However, no FAM response was detected for enriched naturally contaminated chicken samples containing less than 4.3×10^4 CFU/ml of BB. Adding 7% (vol/vol) glycerol (Merck, Darmstadt, Germany) to the PCR mixture reestablished the FAM Ct to values identical to levels seen with dTTP (data not shown).

DISCUSSION

In general, the main advantage of PCR over culture methods is its potential for rapid screening of negative samples. Positive samples, however, still need to be verified by culture to obtain isolates for subtyping, antimicrobial resistance testing, and so on. In contrast to the end point detection by conventional PCR, the real-time PCR provides the opportunity to monitor the accumulation of PCR product during amplification. By generating standard curves from Ct values from samples of known DNA concentrations, information on the linear range of amplification and amplification efficiency of the assay can be determined (17). These features are important when optimizing the amplification, studying PCR inhibition, and investigating the effect of pre-PCR processing. If a real-time PCR method is intended for quantitative measurements, it is a prerequisite to obtain information on both linear range and amplification efficiency in order to ensure correct quantification. In the present study the amplification efficiency of the real-time PCR assay on the ABI-PRISM (89.9%) was similar to that on the RotorGene (90.6%). However, the linear range of amplification was wider on the RotorGene, beginning at 5×10^1 copies of genomic DNA compared to the ABI-PRISM linear range, beginning at 10^3 copies. The PCR results (Fig. 4)

indicate that the method described may be used to estimate the level of *Campylobacter* in flocks or meat products, although exact quantification requires use of PCR directly on samples without any preenrichment. However, the available technology for sample treatment and DNA extraction does not provide us with a detection limit of 1 CFU per 25-g sample, which is required by international standards.

The limitation in current PCR detection is partly due to loss of *Campylobacter* during the DNA extraction step and partly due to the low sample volume (5 μl) tested. The only publication available on quantitative *C. jejuni* PCR testing of nonenriched chicken samples reports the use of a commercial DNA extraction kit on highly contaminated chicken samples from the Chinese market, containing $>10^5$ CFU of *Campylobacter*/ml before enrichment (20). The *Campylobacter* contamination level of chicken flocks and chicken products in countries with more-hygienic production methods is known to be much lower, making enrichment of most samples necessary to obtain PCR-detectable results. Cheng and Griffiths have previously observed that a minimum of 100 *Campylobacter* CFU per ml is necessary for PCR detection by Triton X-100 DNA extraction. Therefore they used a 12-h enrichment step before PCR detection, although they did not test any naturally contaminated samples (5).

The enrichment followed by real-time PCR data presented here indicates that even chicken flocks with low levels of infection would be detected, in particular by the RotorGene platform. The PCR-based method showed the same responses as the culture-based method, indicating that it is as good as the existing "gold standard." Testing the spiked and naturally contaminated samples on RotorGene resulted in lower FAM Ct values (stronger signal) and produced one more positive result than the ABI-PRISM, reflecting a greater sensitivity of this platform.

The terms sensitivity and detection limit are often used interchangeably in diagnostic PCR and real-time PCR. However, both sensitivity and detection limit can be considerably affected by several factors, including presence of inhibitory substances and quality of DNA. Although a given concentration of cells can be detected by a real-time PCR, it will not necessarily be detected in every real-time PCR. For this reason, the detection limit of any PCR and real-time PCR should always be assessed in association with the detection probability to illustrate the reliability of the assay (17).

In conclusion, the real-time PCR method described seems to be sensitive and robust both for detection and quantification in enriched samples. The method is intended for further validation in an international collaborative trial with regard to standardization.

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Comparative, Collaborative, and On-Site Validation of a TaqMan PCR Method as a Tool for Certified Production of Fresh, *Campylobacter*-Free Chickens

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Certified *Campylobacter*-free poultry products have been produced in Denmark since 2002, the first example of fresh (unprocessed and nonfrozen) chickens labeled "*Campylobacter* free." This success occurred partly through use of a 4-hour gel-based PCR testing scheme on fecal swabs. In this study, a faster, real-time PCR approach was validated in comparative and collaborative trials, based on recommendations from the Nordic system for validation of alternative microbiological methods (NordVal). The comparative real-time PCR trial was performed in comparison to two reference culture protocols on naturally contaminated samples (99 shoe covers, 101 cloacal swabs, 102 neck skins from abattoirs, and 100 retail neck skins). Culturing included enrichment in both Bolton and Preston broths followed by isolation on Preston agar and mCCDA. In one or both culture protocols, 169 samples were identified as positive. The comparative trial resulted in relative accuracy, sensitivity, and specificity of 98%, 95%, and 97%, respectively. The collaborative trial included nine laboratories testing neck skin, cloacal swab, and shoe cover samples, spiked with low, medium, and high concentrations of *Campylobacter jejuni*. Valid results were obtained from six of the participating laboratories. Accuracy for high levels was 100% for neck skin and cloacal swab samples. For low levels, accuracy was 100% and 92% for neck skin and cloacal swab samples, respectively; however, detection in shoe cover samples failed. A second collaborative trial, with an optimized DNA extraction procedure, gave 100% accuracy results for all three spiking levels. Finally, on-site validation at the abattoir on a flock basis was performed on 400 samples. Real-time PCR correctly identified 10 of 20 flocks as positive; thus, the method fulfilled the NordVal validation criteria and has since been implemented at a major abattoir.

Human campylobacteriosis is a widespread zoonotic foodborne infection (9). The majority of infections are sporadic and the sources rarely determined (20). Humans can be infected by eating insufficiently cooked meat products (poultry, pork, and beef), by drinking raw milk and polluted water, and by coming into contact with pets (2). A Danish case-control study has identified consumption of undercooked poultry as one of the main causes of human infections (19). In 2003, approximately 35% of Danish broiler flocks were infected with *Campylobacter jejuni* (6).

Rapid detection methods are an important part of any intervention strategy, which may also include separating *Campylobacter*-positive from *Campylobacter*-negative flocks at slaughter. The production of certified, fresh *Campylobacter*-free poultry products has been taking place in Denmark since 2002 and is the world's first example of fresh chickens labeled for sale as being "*Campylobacter* free." This certification has been based on a conventional gel electrophoresis PCR. In general, the superiority of PCR testing lies in its ability to detect the pathogen in cloacal swab samples within the same working shift, making it possible to slaughter chicken flocks with a known *Campylobacter*-negative status before dealing with positive flocks. The purpose of the present study was to validate a newly developed TaqMan PCR method (12) in comparative and collaborative trials according to the recommendations of the Nordic validation organization, NordVal (5). The PCR method used includes an internal amplification control (IAC), reinforcing its value as a diagnostic tool.

The comparative validation was performed against two reference culture methods, applying different combinations of selective enrichment media and selective plating media. To our knowledge, none of the published, noncommercial, real-time PCR methods for the detection of thermotolerant *Campylobacter* have been evaluated through a collaborative trial (17, 21, 22, 23, 24). It is essential for routine application that an analytical method is reliable and consistent. To establish these features, extensive testing of the robustness and performance characteristics of the method is required, preferably through a collaborative trial, including end-use laboratories (10) and on-site on a flock basis.

MATERIALS AND METHODS

Comparative study. The Preston broth was prepared according to the recommendations of the Nordic Committee on Food Analysis (1). Briefly, 25 g of nutrient broth no. 2 (catalog no. CM67; Oxoid, Basingstoke, United Kingdom) was dissolved in 1,000 ml of distilled water, autoclaved for 15 min at 121°C, and, after cooling to 50°C, was combined aseptically with a mixture of 50 ml lysed horse blood (Danish Institute for Food and Veterinary Research [DFVF], Copenhagen, Denmark), 4 ml of modified Preston *Campylobacter* selective supplement (catalog no. SR204E; Oxoid), and 4 ml of *Campylobacter* growth supplement (catalog no. SR084E; Oxoid).

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The Bolton broth was prepared according to the manufacturer's recommendations. Briefly, 13.8 g of Bolton broth (catalog no. CM0983; Oxoid) was dissolved in 500 ml of distilled water, autoclaved for 15 min at 121°C, and, after cooling to 50°C was combined aseptically with a mixture of 25 ml lysed horse blood (DFVF) and one vial of Bolton broth selective supplement (SR0183E) reconstituted as directed.

The modified charcoal cefoperazone deoxycholate agar (mCCDA) was prepared according to the manufacturer's recommendations. Briefly, 45.5 g of *Campylobacter* blood-free selective agar (catalog no. CM0739; Oxoid) was suspended in 1,000 ml of distilled water and brought to boil to dissolve completely. The agar was autoclaved at 121°C for 15 min and, after cooling to 50°C, was combined aseptically with two vials of CCDA selective supplement (catalog no. SR0155; Oxoid) reconstituted twice with 2 ml sterile distilled water.

The Preston agar was prepared according to the manufacturer's recommendations. Briefly, 18.5 g of *Campylobacter* agar base (catalog no. CM0689; Oxoid) was suspended in 475 ml of distilled water and brought to boil to dissolve completely. The agar was autoclaved at 121°C for 15 min, and after cooling to 50°C was combined with a mixture of 25 ml lysed horse blood (DFVF) and one vial of Preston *Campylobacter* selective supplement (catalog no. SR0117; Oxoid) reconstituted with 2 ml of 50/50 acetone/sterile distilled water.

The brain heart infusion medium (BHI) was prepared according to the manufacturer's recommendations and consisted of 37 g/liter BHI (Difco, Sparks, MD) with 5% (vol/vol) calf blood (DFVF) and 0.5% agar added.

***Campylobacter* real-time PCR.** Real-time TaqMan PCR was performed in a RotorGene 3000 (Corbett Research, Australia) in 0.2-ml PCR tubes as described previously (12), except for the addition of 2.0 μl/reaction of 87% pure glycerol (Merck A/S, Denmark) and 1.0 μl/reaction of 12.5 mM dNTP mix with dUTP (Applied Biosystems, Foster City, CA), enabling uracil-*N*-glycosylase treatment to prevent carryover contamination. Each PCR analysis included a positive DNA control, a negative DNA control, a nontemplate control (NTC), and an IAC. The cutoff level and definition of positive/negative responses were exactly as described previously (12). Samples with a threshold cycle (C_T) response below 40 were considered to be positive.

Sampling. As shown in Table 1, the following samples were collected in Denmark: 99 pooled fecal samples on shoe covers from rearing houses, 101 cloacal swab samples from an abattoir, 102 samples of neck skin from an abattoir, and 100 samples of neck skin from the retail sector. The shoe cover, cloacal swab, and neck skin samples from the abattoir originated from flocks at 34 different farms. Approximately half of the samples were taken in the spring, when the prevalence of *Campylobacter* spp. in chicken flocks is expected to be low in Denmark (approximately 20%), and the other half were taken in the early autumn, when the prevalence is expected to be high (approximately 60%). Approximately half of the neck skin samples from the retailers were taken from chicken flocks that were reported to be *Campylobacter* positive by the supplier, and the other half from flocks that had tested negative.

Sample preparation. Fecal samples were collected on disposable shoe covers in the rearing houses and shipped at ambient temperature to DFVF. On arrival, the shoe cover samples were weighed and added to 1:10 (wt/vol) physiological saline and homogenized for 60 s in a stomacher.

The cloacal swab samples were taken at the abattoir from 12 or 13 individual broilers on arrival at the abattoir. The swabs were stored in tightly capped 15-ml plastic tubes with BHI and shipped to DFVF. On arrival, 20 samples were pooled into 1 sample and homogenized manually in 30 ml physiological saline for 60 s. Because of the high background flora in the fecal samples, growth during transportation (one day) was regarded as insignificant to the outcome of the test.

Approximately 40 g of chicken neck skin was cut and pooled into a plastic bag at the slaughter line. The samples were transferred to a stomacher bag, sealed, and shipped on ice packs to DFVF by mail. On arrival, the samples were diluted 1:1 in physiological saline and homogenized for 60 s in a stomacher.

For retail samples, fresh (nonfrozen) chickens were purchased; 40 g neck skin was cut at the laboratory and diluted 1:1 in physiological saline and homogenized for 60 s in a stomacher.

Reference culture methods. The detection of *Campylobacter* spp. was conducted in accordance with the recommendations from the Nordic Committee on Food Analyses (1) and the International Organization for Standardization (3). All samples were enriched in Bolton and Preston broths (1:10) at 42 ± 0.5°C in a microaerobic atmosphere (6% O_2, 7% CO_2, 7% H_2, and 80% N_2) for 24 h before 100 μl was plated onto Preston agar and mCCDA. The agar plates were incubated at 42 ± 0.5°C in a microaerobic atmosphere for 48 h. From the selective agar, five typical thermotolerant *Campylobacter* colonies were selected for verification by an internationally validated gel-based PCR assay for the identification of *Campylobacter jejuni*, *C. coli*, and *C. lari* (15, 16). In the absence of typical colonies, five nontypical colonies were selected for PCR verification.

DNA extraction by magnetic beads. From the fecal sample suspensions (cloacal swabs and shoe covers), 1-ml aliquots were drawn for DNA extraction before enrichment. From the neck skin samples, 1-ml aliquots were drawn for DNA extraction after overnight enrichment in Bolton broth. The samples were centrifuged at 16,000 × *g* for 7 min at 4°C, and DNA extraction was performed on a KingFisher processor (Thermo Lab Systems, Helsinki, Finland) using an automated, magnetically based separation and DNA isolation kit for blood, cells, and tissue (Thermo Lab Systems) as specified by the manufacturer. Briefly, the sample pellet was resuspended in lysis buffer and transferred to a 96-well plate (Thermo Lab Systems) containing magnetic particles, washing buffers, and elution buffer. The DNA extraction program consisted of two salt-buffer washing steps and two alcohol-buffer washing steps, followed by a final elution step (for a detailed protocol, see the Rapid Diagnostic Group website [http://www.pcr.dk/Innovations_pcr/innovations_pcr_startside.htm]). A total of 5 μl of the extracted DNA was used as the template in the real-time PCR.

Statistical data analysis. The comparative validation study included three test characteristics: relative accuracy, sensitivity, and specificity (5) (see Table 2). The relative accuracy is defined as the degree of correspondence between the response obtained by the alternative method and the reference method on identical samples, as follows: (PA + NA + FP) × 100/(PA + NA + TP + FN + FP), where PA refers to positive agreement, NA to negative agreement, FP to false positives, TP to true positives, and FN to false negatives. The relative sensitivity is defined as the ability of the alternative method to detect the target microorganism compared to the reference method, as follows: (PA + TP) × 100/(PA + FN). The relative specificity is defined as the ability of the alternative method not to detect the target microorganism when it is not detected by the reference method, as follows: (NA × 100)/(NA + FP).

To compare the performances of Bolton and Preston broths, the number of positive responses obtained from each was subtracted, giving one difference for each combination of sample type and agar type. The Wilcoxon signed-rank test with a continuity correction was applied to these differences to test whether the two enrichment broths differed significantly from each other (14). The calculations where performed using Splus software, professional edition version 6.1.

Collaborative trial. A collaborative trial involving nine national laboratories was performed to evaluate the robustness and reproducibility of the real-time PCR method testing identical samples.

The collaborative trial was designed and conducted according to the recommendations from NordVal (5). The nine participating laboratories received pellets from 18 coded 1-ml samples, including 6 chicken neck skin samples, 6 shoe cover samples, and 6 cloacal swab samples (see Table 3). The samples were spiked in duplicate with *C. jejuni* CCUG 11284 at three levels, making it possible to assess the usefulness of the method at various infection levels. The shipment included a positive DNA control (1 μg/ml *C. jejuni* CCUG 11284) and a negative DNA control (1 μg/ml *Arcobacter butzleri* CCUG 30485), a ready-to-use PCR mixture with added IAC, and reagents for the magnetically based DNA extraction. To minimize any interlaboratory variability (not attributable to the method performance), we supplied all the reagents necessary. Each participant received a detailed protocol describing the DNA extraction, real-time PCR setup, real-time PCR run, and data analysis and a reporting form to record the obtained PCR results to return to DFVF. The participants were also asked to return a file containing the real-time PCR runs.

A second collaborative trial, comprising eight participating laboratories, was subsequently performed only on shoe cover samples. The second trial was performed exactly as the first one, except for using a modified DNA extraction protocol, with an increased amount of paramagnetic particles.

Samples. The samples for the collaborative trial were prepared as described above ("Sample preparation"). Regarding the neck skin samples, one broth was left unspiked, one was spiked with 1 to 10 CFU/100 ml, and one with 10 to 100 CFU/100 ml, and incubated at 42 ± 0.5°C for 24 h in a microaerobic atmosphere. After the enrichment, 1-ml aliquots were drawn and centrifuged at 16,000 × *g* for 7 min at 4°C. The supernatant was discarded, and the pellet kept at −80°C until shipped on ice to the trial participants.

From both shoe cover and cloacal swab samples, 1-ml aliquots were drawn, spiked with 0, 100 to 500, or 1,000 to 2,000 CFU/ml, and centrifuged at 16,000 × *g* for 7 min at 4°C. The supernatant was discarded and the pellet kept at −80°C until shipped on ice to the trial participants.

The *Campylobacter* status of all samples was confirmed at DFVF by the reference culture method according to International Organization for Standardization publication no. 10272-1 (3) and Nordic Committee on Food Analyses publication no. 119 (1) prior to and after spiking. The stability of the samples was examined using real-time PCR (12) immediately after spiking, prior to commencement of the collaborative trial, and during the period of analysis, to verify

TABLE 1. Samples from the chicken production chain with positive isolates of thermotolerant *Campylobacter*

Sample source	No. of samples	No. of *Campylobacter*-positive samples in indicated medium on indicated plate type				
		Total[a]	Bolton broth		Preston broth	
			Preston agar	mCCDA	Preston agar	mCCDA
Shoe covers	99	22	21	22	20	21
Cloacal swabs	101	44	40	42	37	43
Neck skins from:						
Abattoir	102	46	46	46	42	46
Retailers	100	57	57	57	57	57
Total	402	169	164	167	156	167

[a] Total number isolated from at least one of the four agar plates.

TABLE 2. Comparison of the results obtained by the real-time PCR and the reference culture methods

Matrix	No. of samples with indicated result[a]						% Value[b]		
	PA	NA	FN	TP	FP	Total	Accuracy	Sensitivity	Specificity
Shoe covers	18	73	4	0	4	99	96	82	95
Cloacal swabs	39	55	5	0	2	101	95	88	96
Neck skins from:									
Abattoir	46	55	0	0	1	102	100	100	98
Retailers	57	43	0	0	0	100	100	100	100
Total	160	226	9	0	7	402	98	95	97

[a] PA, positive agreement; NA, negative agreement; TP, true positive; FN, false negative; FP, false positive.
[b] See Materials and Methods for accuracy, sensitivity, and specificity formulas.

the continued detection of *Campylobacter*. The possible detrimental effect of shipping time at ambient temperature on the real-time PCR results was investigated, and no detrimental effect was found.

Participating laboratories. At the participating laboratories, DNA was extracted from the samples by using the Magnesil KF genomic system (Promega, Madison, WI) on an automated DNA extraction platform of the laboratories choosing but following the KingFisher protocol described above. In the second trial, the amount of paramagnetic particles was increased from 20 μl/sample to 75 μl/sample. A total of 5 μl purified DNA was used as the template in the real-time PCR.

Real-time PCR at the participating laboratories was performed on a Mx3000 or Mx4000 real-time PCR system (Stratagene, La Jolla, CA), ABI-PRISM 7000 or 7900 (Applied Biosystems, Foster City, CA), RotorGene 3000 (Corbett Research, Mortlake, Australia), or an iCycler thermal cycler (Bio-Rad, Hercules, CA). The participating laboratories were asked to use the blank (NTC), the process blank (a *Campylobacter*-negative sample processed throughout the entire protocol), and the negative control to assign the cutoff line and report back the C_T values.

Data analysis. The test reports and the real-time PCR analyses from the participating laboratories were carefully evaluated on return to the expert laboratory, and the results were approved for inclusion in the statistical analysis, unless they fell into at least one of the following two categories: (i) obvious performance deviation from the protocol and (ii) presence of target amplicons in the negative control results, indicating cross contamination at the participating laboratory.

The results obtained in the collaborative trial were analyzed according to the recommendations from NordVal (5). The relative specificity was calculated for the unspiked samples by the following equation: $(1 - [FP/N-]) \times 100\%$, where $N-$ refers to the total number of unspiked samples. The relative sensitivity was calculated for each level of spiking by the following equation: $(TP/N+) \times 100\%$, where $N+$ refers to the number of spiked samples. The relative accuracy was calculated for all levels of spiking by the following equation: $([PA + NA + FP]/N) \times 100\%$, where N refers to the number of samples tested.

On-site validation on flock basis at abattoir. At a major abattoir, the real-time PCR method was validated against a routinely used gel-based PCR method approved previously by the Danish authorities to monitor production. The two methods were performed in parallel on 400 pooled cloacal swab samples collected from 20 chicken flocks. Each cloacal swab sample was a pool of 25 swabs. Thus, 20 pooled samples represented 500 chickens per flock. The samples giving a positive signal had C_T values between 17 and 39. All samples giving negative signals had C_T values of >40 (see Table 5).

RESULTS

Comparative trial. Out of 402 samples, 169 were found to be culture positive by at least one of the detection methods used (Table 1). The single highest number of positive samples was obtained by the combination of selective enrichment in Bolton or Preston broth followed by isolation on mCCDA, resulting in 167 positive samples. The combination of Bolton broth and Preston agar was slightly less effective (164 positive samples). Applying Preston broth followed by Preston agar resulted in 156 samples being found positive. The obtained P value was 0.32, indicating no significant difference between the Bolton and Preston broths.

A total of 167 out of 402 samples gave positive results by real-time PCR, compared to a total of 169 by the culture methods. Nine false-negative and 7 false-positive results were obtained by the real-time PCR method. This resulted in a relative accuracy of 98%, a relative sensitivity of 95%, and a relative specificity of 97% (Table 2).

Collaborative trial. In agreement with the predefined criteria, results from three laboratories were excluded because of obvious deviation from the protocol. The results from the remaining laboratories were accepted; thus, the final statistical analysis was performed on six sets of results (Table 3).

The relative specificity, sensitivity, and accuracy were 100% in the testing of neck skin samples. In the testing of cloacal swab samples, a relative specificity of 100% was obtained. A relative sensitivity of 92% was achieved for cloacal swab samples spiked with 100 to 500 CFU/ml, and 100% for the samples spiked with the higher levels, i.e., 1,000 to 2,000 CFU/ml (Table 4).

The real-time PCR method failed to detect any of the shoe cover samples, regardless of the level of spiking, except for one positive signal obtained from a sample spiked with 100 to 500 CFU/ml.

In the second collaborative trial on shoe cover samples, results from all eight participating laboratories were included in the statistical analysis. Only results from six laboratories are shown in Table 3. The relative specificity, sensitivity, and accuracy for shoe cover samples were 94%, 100%, and 100%, respectively (Table 4).

Validation at abattoir. Table 5 shows the real-time PCR results obtained, compared to those from a routinely used gel-based PCR approved by the Danish authorities. The real-time PCR was performed at the testing laboratory of one of the major poultry producers in Denmark. The real-time PCR method correctly identified 10 out of 10 *Campylobacter*-positive chicken flocks and 10 out of 10 *Campylobacter*-negative chicken flocks.

TABLE 3. Collaborative trial: real-time PCR results for the detection of thermotolerant *Campylobacter* spp. in spiked samples from chickens[a]

Level of spiking[b]	C_T values for replicates from indicated source (by participant no.)																	
	Neck skins						Shoe covers						Cloacal swabs					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
None	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 29	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40	40, 40
Low	35, 34	26, 25	33, 32	27, 26	27, 26	28, 28	26, 26	25, 26	26, 26	20, 22	27, 26	28, 27	38, 33	28, 28	39, 40	30, 31	36, 38	31, 31
High	34, 29	20, 22	30, 29	22, 23	21, 21	23, 23	24, 23	23, 23	23, 23	18, 18	24, 24	25, 23	35, 31	24, 25	34, 36	26, 26	27, 29	29, 29

[a] This table includes only results from participants that were not excluded due to obvious deviation from the trial protocol.
[b] Low, 1 to 10 CFU for neck skin and 100 to 500 CFU for shoe covers and cloacal swabs; high, 10 to 100 CFU for neck skin and 1,000 to 2,000 CFU for shoe covers and cloacal swabs.

DISCUSSION

Enrichment in selective broth will always be a compromise between the inhibition of competitive flora and the recovery and growth of the target microorganism. The results of the present study did not detect any difference ($P = 0.32$) in the ability of Preston and Bolton broth to support growth of *Campylobacter.* Martin et al. reached the same conclusion, testing 100 samples of chicken meat, sausage meat, pig offal, unpasteurized milk, and untreated water (18). However, these results differ from the findings of Baylis et al., who found Bolton broth superior to Preston broth, testing 100 raw foods, including chicken carcass, chicken meat, chicken liver, turkey, duck, beef, lamb liver, and pork sausage meat (7). In opposite findings, Borck et al. reported that Preston broth was better in tests of 41 turkey neck skin samples enriched in Preston broth and *Campylobacter* enrichment broth (same formula as Bolton broth) (8). Finally, Josefsen et al. have shown in a comprehensive study that Bolton broth and Preston broth equally support the growth of *C. jejuni*, while Preston broth was less effective in supporting the growth of *Campylobacter coli* (13). These differences in results can be attributed to the matrices and the background flora of the samples; thus, the two enrichment broths can be used equally for routine testing.

Comparative study. In the comparative study, the divergence in the number of negative results between the culture method and real-time PCR could be ascribed to the fact that in the present study, the fecal samples were enriched in Bolton and Preston broths for 24 h and consequently grown to a high concentration of *Campylobacter* spp. in the culture method. Real-time PCR, however, was performed directly on the fecal samples without any preceding enrichment. Seven samples were identified as positive by real-time PCR but were identified as negative using the culture method. This difference may be attributable to the presence of *Campylobacter* spp. that were viable but not culturable or dead in these samples.

Collaborative study. In the first collaborative study, complete agreement between the real-time PCR method and the microbiological reference method was obtained for all test characteristics for neck skin samples. Compared to the reference culture method, real-time PCR detected 11 out of the 12 cloacal swab samples spiked with the target microorganism at a low level, corresponding to a 92% relative sensitivity. However, the level of thermotolerant *Campylobacter* in fecal samples from infected chicken flocks is usually in the range of 4 to 8 $\log_{10}$ CFU/g feces and will most probably be between 5.5 and 6.5 $\log_{10}$ CFU/g feces (22). The number of CFU to be analyzed in a 1-ml sample by the real-time method is estimated to be 2×10^4 to 2×10^5 CFU.

TABLE 4. Validation parameters obtained by the real-time PCR method on spiked chicken samples based on the results obtained in the collaborative trial

Matrix	% Value for indicated level of spiking[a]					
	Specificity (no spiking)	Sensitivity		Accuracy		
		Low	High	No spiking	Low	High
Neck skins	100	100	100	100	100	100
Shoe covers	94	100	100	100	100	100
Cloacal swabs	100	92	100	100	92	100

[a] Low, 1 to 10 CFU for neck skin and 100 to 500 CFU for shoe covers and cloacal swabs; high, 10 to 100 CFU for neck skin and 1,000 to 2,000 CFU for shoe covers and cloacal swabs.

TABLE 5. On-site results of the real-time PCR method on cloacal swabs, validated against an approved gel-based PCR[a]

Flock no.	No. of samples positive by gel PCR/total no. of samples	Real-time PCR	
		No. of samples positive/total no. of samples	C_T value(s)
Campylobacter-positive flocks			
1	19/20	19/20	27–31
2	20/20	20/20	18–30
3	20/20	20/20	17–23
4	16/20	20/20	18–30
5	20/20	20/20	17–27
6	1/20	1/20	32
7	20/20	20/20	18–22
8	8/20	11/20	23–39
9	20/20	20/20	18–25
10	19/20	19/20	17–24
Campylobacter-negative flocks			
11	0/20	0/20	>40
12	0/20	0/20	>40
13	0/20	0/20	>40
14	0/20	0/20	>40
15	0/20	0/20	>40
16	0/20	0/20	>40
17	0/20	0/20	>40
18	0/20	0/20	>40
19	0/20	0/20	>40
20	0/20	0/20	>40

[a] The microbiological status of the flocks was confirmed previously by a reference culture method.

The considerable difference in the C_T values observed among the participating laboratories (Table 3) for the same samples can be attributed to variation in the sensitivity of the real-time PCR platforms used. It has been shown that transferring a PCR method from one type of real-time instrument to another can result in a substantial shift in C_T values (12).

In the first collaborative trial, the real-time PCR method failed to detect any of the shoe cover samples regardless of the level of spiking. Detection of *C. jejuni* in the shoe cover samples was possible at our laboratory at both low and high spiking levels. Posttrial investigations have shown that the reason for this discrepancy lies with the DNA extraction procedure. The extraction protocol provided to the participating laboratories was modified for compatibility with several DNA extraction platforms, and this approach resulted in a markedly reduced amount of total DNA recovery. The shoe cover samples can be more PCR inhibitory and yield less DNA than the other sample matrices, and it is likely that these features were the cause of the absence of positive signals.

In the second collaborative trial, the amount of paramagnetic particles was more than tripled, rectifying the problem with DNA extraction from this matrix. Shoe cover samples contain substantial amounts of extraneous material, and the results indicate that clotting of the magnetic particles hindered the absorption of DNA.

A relative specificity of 94% was obtained for shoe cover samples in the second collaborative trial, since one laboratory obtained a positive signal from one of the nonspiked samples (Table 3). This unexpected result could be due to cross contamination during DNA extraction or miscoding of the sample at DFVF.

The samples tested in the collaborative study were not naturally infected. Because the concentrations of the target microorganism in naturally infected samples are unknown, it would have been difficult to assess the method performance on samples with low levels of thermotolerant *Campylobacter* spp.

Validation at abattoir. The on-site, flock-based validation of real-time PCR at the abattoir against an existing conventional gel-PCR method was successful. It should be noted that the *Campylobacter* status of the flocks had been previously determined by a reference culture method (1) on shoe cover samples from the rearing houses. This practice allows the poultry manufacturer to perform separated slaughtering. Pooled cloacal swab samples are taken out immediately after the killing and tested to determine the *Campylobacter* status of the chicken flocks and subsequently to label and mark the chickens as being *Campylobacter* free in accordance with Danish regulations. The regulation states that if one or more of the 20 pooled samples are *Campylobacter* positive, the whole flock should be regarded as being positive. Although some minor variations were seen in single samples, the results from the conventional gel-based PCR and the microbiological history of the flocks confirmed the real-time PCR results, emphasizing that the faster and less work demanding real-time PCR would be a practical alternative to the gel-based PCR.

In addition, the combination of an automated DNA extraction and the closed system of the real-time PCR provides a faster and less work-intensive method with a minimized risk of contamination compared to the gel-based PCR. Furthermore, the real-time PCR method includes the dUTP–uracil-*N*-glycosylase system, minimizing the risk of carryover contamination. The PCR reagents used in the method can be mixed in advance, distributed in smaller quantities, and frozen at −20°C for up to 6 months and be ready to use. These features are a major benefit for on-site use of the test. The method is an open-formula technique, i.e., the reagents and target gene, etc., are known, in contrast to commercial kits.

In conclusion, the real-time PCR method complied with the criteria for the validation of alternative microbiological methods and has been approved by NordVal as an alternative method for detection of thermotolerant *Campylobacter* spp. in chicken samples. The method is currently implemented for use in separated-slaughtering practice by the leading poultry producers in Denmark as part of a risk management program, and for the certified production of *Campylobacter*-free chicken.

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Optimization of a 12-Hour TaqMan PCR-Based Method for Detection of *Salmonella* Bacteria in Meat†▽

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We developed a 12-h *Salmonella* detection method, based on 8 h of preenrichment, followed by automated DNA extraction and a sensitive real-time PCR. The method was optimized to obtain the highest possible yield of cells and DNA. The growth of different *Salmonella* strains in various preenrichment media and the effects of adding growth-promoting and selective reagents were explored, taking into account their PCR compatibility. The effects of (i) analyzing larger volumes (1 to 5 ml) from preenriched samples and introducing wash steps prior to DNA extraction, (ii) regulating the amount of paramagnetic particles (increasing it from 60 to 90 μl) in the DNA extraction, (iii) eluting the DNA in reduced volumes (25 or 50 μl rather than 100 μl), and (iv) increasing the PCR template volume (from 5 to 20 μl) were investigated. After 8 h of preenrichment, buffered peptone water yielded the highest number of salmonellae. When analyzing minced meat samples, positive effects of increasing the initial sampling volume from 1 to 5 ml and increasing the amount of paramagnetic particles to 90 μl were observed. However, washing the pellet and eluting the DNA in reduced volumes (25 and 50 μl) had no positive effects and resulted in decreased reproducibility. Increasing the amount of PCR template DNA from 5 to 20 μl improved the threshold cycle value by approximately 2. The improved 12-h PCR method was successfully compared to a reference culture method with 100 minced meat and poultry samples, with a relative accuracy of 99%, a relative sensitivity of 98%, and a relative specificity of 100%.

Bacteriological detection of *Salmonella* in foods and environmental samples is costly, laborious, and time-consuming, requiring up to 5 days to obtain a confirmed result. Thus, rapid and cost-effective detection of *Salmonella* is of major interest to the food industry and the public. Real-time PCR technology offers several advantages compared to classical bacteriology in terms of speed, detection limit, potential for automation, and cost (17, 24). However, it is essential that new PCR methods be reliable and robust. They have to comply with legislation on microbiological criteria for foodstuffs and be able to detect as few as one *Salmonella* bacterium per 25-g sample. They should be validated against reference culture methods, and last, but not least, they should be sufficiently robust to be transferred from the expert laboratory to end users.

Several PCR-based methods for the detection of *Salmonella* in foodstuff have been published. Most of these methods operate with preenrichment periods of 16 to 24 h, followed by DNA purification and gel-based or real-time PCR (8, 11, 13, 16, 18, 34). Only a few of them report reduced preenrichment times of 6 to 12 h (1, 12, 27). Both Ellingson et al. (12) and Agarwal et al. (1) reported a preenrichment period of only 6 h for the detection of *Salmonella* by PCR. In a study by Ellingson et al. (12), the results obtained by real-time PCR correlated 100% with a reference culture method. However, for both studies, the samples analyzed were inoculated with at least 1 CFU/g (not per 25 g) food sample; thus, it is questionable if these methods can meet the legislative demand of detection of 1 CFU/25-g sample. In a recent study by Myint et al. (27) five *Salmonella*-positive chicken samples were subjected to PCR after 2 to 18 h of preenrichment, with a sampling interval of 2 h. Even though two out of the five samples were detectable by PCR after 8 h of preenrichment, it required 18 h of preenrichment for all five samples to be detected.

The present study describes the development and optimization of a 12-h *Salmonella* analysis for the meat industry, enabling a faster release of *Salmonella*-free fresh meat and meat products. The method is based on a shortened preenrichment period combined with increased detection sensitivity in a real-time PCR.

Because of low levels of *Salmonella* in meat from subclinically infected herds, a preenrichment step is usually included prior to PCR. The preenrichment was followed by a TaqMan PCR assay including an internal amplification control (IAC) (25). However, the shortened preenrichment period of only 8 h did not produce *Salmonella* counts that could be detected consistently by this PCR. For this reason, critical steps throughout the method were optimized in order to obtain the highest possible yield of cells and DNA recovery after automated DNA extraction. In addition, the detection limit of the PCR method was optimized by implementing the locked nucleic acid (LNA) technology (30). LNA probes have a higher melting temperature than TaqMan probes because the LNA modifications provide stronger hybridization between double-stranded DNAs and are reported to be more sensitive (28).

Besides evaluating alternative preenrichment broths, optimization of pre-PCR treatment was attempted by increasing the sampling volume and introducing washing steps prior to DNA extraction. The DNA extraction protocol used in this

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study has been shown to be a promising method for extracting bacterial DNA from other matrices (23). The DNA loss in this method was evaluated, and the method was optimized with regard to the amounts of paramagnetic particles and elution buffer. Finally, the effect of increasing the volume of template DNA was studied.

The improved 12-h PCR method was compared to the reference culture method for *Salmonella* from the Nordic Committee on Food Analysis (NMKL no. 71; reference 3) with 50 artificially inoculated samples of minced pork meat and 50 artificially inoculated poultry samples.

MATERIALS AND METHODS

Optimization of growth conditions. In the experiments described below, all of the samples and media were preheated to 37°C prior to preenrichment.

The first step was to confirm if shaking during preenrichment had any beneficial effect on the number of salmonellae present after 8 h (29). Overnight cultures of four of the *Salmonella* strains most frequently isolated from pork were prepared in buffered peptone water (BPW; Oxoid, Basingstoke, United Kingdom). The number of CFU per milliliter was determined by plating 10-fold dilution series on blood agar (Statens Serum Institute, Copenhagen, Denmark) in duplicate.

Salmonella enterica serovar Typhimurium CCUG 31939, *S. enterica* serovar Enteritidis CCUG 32352, *S. enterica* serovar Dublin, and *S. enterica* serovar Infantis (in-house collection) were inoculated at a level of 1 to 10 CFU into 100 ml of BPW in quadruplicate and incubated at 37°C. Half of the preenrichments were shaken (60 rpm, Certomat U; B. Braun Biotech International, Göttingen, Germany), and half were incubated without shaking. The numbers of salmonellae in the preenrichments were determined by plating on blood agar after 6, 8, and 24 h of incubation.

The second step was to examine whether alternative nutrient media would be superior to BPW in supporting the growth of *Salmonella* and if these were PCR compatible, i.e., not inhibiting the PCR. One-hundred-milliliter volumes of brain heart infusion (BHI) broth (Oxoid) and tryptone soya broth (TSB; Becton Dickinson, Franklin Lakes, NJ) were inoculated in duplicate with 1 to 10 CFU of each aforementioned *Salmonella* strain and incubated at 37°C. The number of salmonellae in the preenrichments was determined by plating on blood agar after 6, 8, and 24 h of incubation. To test the PCR inhibition of the media, an overnight culture containing 1.9×10^9 CFU/ml was diluted in BPW, BHI, and TSB to 10^{-5} to 10^{-8} and the DNA was extracted and analyzed in duplicate in the PCR assay.

Growth-promoting reagents. The third step in the optimization process was to study the effect of adding sodium pyruvate to the preenrichment media (26). One-hundred-milliliter volumes of BPW containing 0, 0.2, and 0.4 g/liter sodium pyruvate (Sigma) were inoculated in duplicate with 1 to 10 CFU of each *Salmonella* strain and incubated at 37°C. The numbers of salmonellae in the BPW were determined by plating on blood agar after 6, 8, and 24 h of incubation.

The effect of adding egg yolk to the preenrichment media was investigated. *S. enterica* serovar Typhimurium CCUG 31939 and *S. enterica* serovar Infantis (in-house collection) were inoculated at a level of 1 to 10 CFU into 100 ml BPW containing 0.5, 1.0, and 5.0% egg yolk (Oxoid) and incubated at 37°C. The number of salmonellae in the BPW was determined by plating on blood agar after 8 h of incubation.

Selective reagents. In order to suppress competitive flora and thereby improve the growth conditions for *Salmonella*, the effect of adding a range of different selective reagents to the BPW was investigated. To BPW were added novobiocin (20, 50, and 100 mg/liter; Fluka, Buchs, Switzerland), brilliant green (10, 20, and 50 mg/liter; Fluka), malachite green oxalate salt (50, 100, and 250 mg/liter, Fluka), tergitol 4 (1, 2, and 4 ml/liter; Fluka), sodium deoxycholate (2.5, 5, and 7.5 g/liter; Fluka), and finally sulfamandelate supplement (1, 2, and 3 vials/liter; Oxoid). Samples of minced pork meat (10 g), frozen at −18°C and thawed, were transferred to 90 ml of BPW with the selective reagents added and inoculated with 1 to 10 and 10 to 100 CFU/g sample by using freeze-stressed *S. enterica* serovar Typhimurium CCUG 31939 and *S. enterica* serovar Infantis (in-house collection). Stressed cells were prepared from a BHI culture grown at 37°C for 20 to 24 h and frozen at −18°C. Before use, both minced meat samples and freeze-stressed cells were thawed at 4°C. The samples were incubated at 37°C for 20 h. Aliquots for PCR were drawn after 6, 8, and 20 h. DNA extraction was performed prior to analysis by the PCR assay (see below).

Automated DNA extraction. One-, 2-, and 5-ml aliquots were drawn from the preenrichments for DNA extraction. The aliquots were centrifuged at $3,000 \times g$ for 5 min at 4°C, and DNA extraction was performed with a KingFisher (Thermo Labsystems, Helsinki, Finland) and a DNA isolation kit for blood, stool, cells, and tissue (Magnesil KF, Genomic System; Promega) as specified by the manufacturer. Briefly, the sample pellet was resuspended in lysis buffer and transferred to a 96-well plate (Thermo Labsystems) containing paramagnetic particles, washing buffers, and elution buffer. The DNA extraction program consisted of two salt buffer washing steps and two alcohol buffer washing steps, followed by a final elution step (for a detailed protocol, see InnovationsPCR at www.foodpcr.com). Five to 20 μl of the extracted DNA was used as the template in the PCR.

TaqMan PCR. A TaqMan real-time PCR method, targeting a region within the *ttrRSBCA* locus required for tetrathionate respiration, for the specific detection of *Salmonella* was set up (adopted from reference 25, with the following modifications). The PCR was performed on an Mx3005P (Stratagene, La Jolla, CA) in a total reaction volume of 25 μl, consisting of 1.5 U of *Tth* DNA polymerase (Roche Applied Science, Mannheim, Germany), 2.5 μl of 10× PCR buffer for *Tth* DNA polymerase (Roche Applied Science), 500 μM deoxynucleoside triphosphate blend with dUTP (Applied Biosystems, Foster City, CA), 4.0 mM $MgCl_2$ (Roche Applied Science), 8% pure glycerol (Merck, Darmstadt, Germany), 1 g/liter bovine serum albumin (Roche Applied Science), 2% dimethyl sulfoxide (Sigma, Steinheim, Germany), 240 nM both LNA target probe (6-FAM [6-carboxyfluorescein]–CG+ACGGCG+AG+ACCG-BHQ1; Sigma-Proligo, Paris, France) and an IAC probe (JOE-CACACGGCGACGCGAAC GCTTT-BHQ1; MWG Biotech, Ebersberg, Germany), and 5 μl of purified DNA. The cycle temperature profile was initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 65°C for 60 s, and 72°C for 30 s. Fluorescence measurements were obtained online and analyzed with the MxPro-Mx3005P software (version 3.00). The threshold was assigned by using the software option background-based threshold; i.e., the standard deviation of all amplifications was determined from cycle 5 to cycle 9, and this value was multiplied by a background sigma multiplier of 10. Each PCR run included three positive DNA controls (*S. enterica* serovar Typhimurium 51K61; Institute for Reference Material and Measurements [IRMM], Geel, Belgium) at final concentrations of 0.5, 0.05, and 0.005 ng/PCR tube, a nontemplate control (only the master mix and PCR grade water), and a negative DNA control (*Escherichia coli* O157; IRMM) at a concentration of 5 ng/PCR tube.

Optimization of sample preparation. The effects of analyzing larger volumes from BPW and introducing washing of the pellet prior to DNA extraction were investigated. A 25-g sample of minced pork meat was inoculated with 1 to 10 CFU of *S. enterica* serovar Livingstone (in-house collection), transferred to 225 ml of BPW, and incubated at 37°C for 8 h. One-, 2-, and 5-ml aliquots were drawn (eight replicates). DNA was extracted from half of the replicates directly, and pellets from the remaining replicates were washed before DNA extraction; i.e., twice they were centrifuged at $3,000 \times g$ for 5 min and the pellet was resuspended in 1 ml of physiological saline. After DNA extraction, replicates were analyzed in the PCR.

Loss of DNA during extraction. The amount of DNA lost in the extraction procedure was evaluated by comparing C_T (threshold cycle) values of samples containing a known amount of reference DNA before and after automated DNA extraction. As shown in Table 1, the experiment was designed so that theoretically equivalent amounts of DNA were analyzed in the PCR. Four samples were prepared from reference DNA (*S. enterica* serovar Typhimurium 51K61; IRMM) and 1× Tris-EDTA buffer to final concentrations of 0.1, 0.05, 0.01, and 0.005 ng/μl. The samples were analyzed in duplicate in the PCR before and after automated DNA extraction.

In the same experimental setup, the effect of regulating the amount of paramagnetic particles was investigated. The DNA was extracted in triplicate from samples containing the aforementioned concentrations of DNA by using 60, 75, and 90 μl of paramagnetic particles, respectively. The samples were subsequently analyzed in replicate in the PCR.

Increasing the concentration of DNA. The effect of reducing the volume of elution buffer to increase the DNA concentration was investigated. A 25-g sample of minced pork meat was inoculated with 1 to 10 and 10 to 100 CFU of *S. enterica* serovar Typhimurium CCUG 31939, transferred to 225 ml of BPW, and incubated at 37°C for 8 h. One-milliliter aliquots were drawn (nine replicates from each preenrichment), and the DNA was extracted from the replicates with 100, 50, and 25 μl of elution buffer, respectively. The replicates were analyzed in the PCR.

Increasing the PCR template DNA volume. The effect of increasing the PCR template volume was investigated. A 25-g sample of minced pork meat was inoculated with 1 to 10 or 10 to 100 CFU of *S. enterica* serovar Typhimurium CCUG 31939, transferred to 225 ml of BPW, and incubated at 37°C for 8 h.

TABLE 1. Loss of DNA in the extraction procedure

Treatment and DNA concn (ng/μl) in PCR mixture	Amt (ng) of DNA entering KingFisher	Elution vol (μl), KingFisher	C_T value[a]	
			Salmonella/FAM	IAC/HEX
Direct PCR				
0.1			20.5	30.6
0.1			20.3	31.4
DNA extraction				
0.1[b]	8	80	25.6	30.1
0.1[b]	8	80	25.5	30.3
Direct PCR				
0.05			20.6	29.8
0.05			20.0	30.2
DNA extraction				
0.05[b]	4	80	25.0	30.1
0.05[b]	4	80	25.5	30.1
Direct PCR				
0.01			23.8	30.3
0.01			23.0	31.0
DNA extraction				
0.01[b]	0.8	80	28.7	29.8
0.01[b]	0.8	80	27.6	29.7
Direct PCR				
0.005			22.7	30.3
0.005			22.3	30.4
DNA extraction				
0.005[b]	0.4	80	26.9	29.9
0.005[b]	0.4	80	27.0	30.2

[a] C_T values obtained in PCR from samples with similar DNA concentrations subjected directly to PCR and following DNA extraction.

[b] Estimated DNA concentration if the extraction procedure was 100% efficient.

One-milliliter aliquots were drawn (three replicates from each preenrichment). DNA was extracted and subsequently analyzed in the PCR with 5 and 10 μl of template DNA in a total volume of 25 μl of master mix and 20 μl of template DNA in a total volume of 50 μl of master mix.

Validation against a reference culture method. The final 12-h PCR method was compared to the reference culture method for *Salmonella* from the Nordic Committee on Food Analysis (NMKL no. 71; reference no. 3) with 100 artificially inoculated minced meat and poultry neck skin samples. As the prevalence of *Salmonella*-positive pork meat samples is 1 to 2% and that of *Salmonella*-positive broiler meat samples is 1.7% at the moment (5), a statistically valid study would require a very large number of samples. For this reason, the comparison was performed with samples artificially inoculated with *Salmonella* in the exponential growth phase. This alternative to naturally contaminated samples is in compliance with international guidelines (6, 7).

Twenty-five grams of *Salmonella*-free fresh minced pork meat was transferred to 225 ml of BPW (37°C). Nine samples were inoculated with 1 to 10 CFU of *S. enterica* serovar Typhimurium, 10 were inoculated with 1 to 10 CFU of *S. enterica* serovar Livingstone, and 14 were inoculated with 10 to 100 CFU of *S. enterica* serovar Livingstone. The remaining 17 samples were left uninoculated.

Twenty-five grams of poultry neck skin was cut into small pieces and transferred to 225 ml of BPW (37°C). Fifteen samples were inoculated with 1 to 10 CFU of *S. enterica* serovar Enteritidis, and 14 were inoculated with 1 to 10 CFU of *S. enterica* serovar Typhimurium. The remaining samples were left uninoculated.

All of the samples were preheated to 37°C and homogenized by hand for 20 s. After 8 h of preenrichment at 37°C, 5-ml aliquots were drawn for DNA extraction with 75 μl of paramagnetic beads, followed by a PCR with 10 μl of the extracted DNA as the template.

The enrichment was thereafter continued for up to 24 h according to NMKL no. 71 (3). The next day, 100 μl was transferred to 10 ml of Rappaport-Vassiliadis soy peptone (Oxoid) broth preheated to 37°C. The Rappaport-Vassiliadis soy peptone broth was incubated at 41.5°C for 24 h and inoculated onto the surface of the selective plating media xylose lysine deoxycholate (Oxoid) and Rambach (Merck). The plates were incubated at 37°C for 24 h, and presumptive colonies were transferred to 5% blood agar plates (Statens Serum Institute) and confirmed by API 20E (BioMérieux, Marcy l'Étoile, France) and by serotyping.

Statistical analysis. A paired-sample *t* test was performed according to Campbell (10) on the data obtained by comparing preenrichments that were shaken and those that were not shaken. The data obtained by comparing the three preenrichment broths (BPW, BHI, and TSB) and adding growth-promoting reagents to the preenrichments were analyzed in a single-factor one-way analysis of variance according to Campbell (10), with an α of 0.05.

When comparing the improved 12-h PCR method to the reference culture method, the test characteristics relative accuracy (AC), sensitivity, and specificity were evaluated (7). AC is defined as the degree of correspondence between the responses obtained by the PCR method and the reference culture method with identical samples, as follows: $(PA + NA) \times 100/(PA + NA + PD + ND)$, where PA refers to positive agreement, NA is negative agreement, PD is positive deviation, and ND is negative deviation. Sensitivity is defined as the ability of the PCR method to detect the target compared to the reference culture method, as follows: $PA \times 100/(PA + FN)$, where FN refers to false negatives. Specificity is defined as the ability of the PCR method not to detect the target when it is not detected by the reference culture method, as follows: $(NA \times 100)/(NA + FP)$, where FP refers to false positives.

RESULTS

Optimization of growth conditions. Shaking the preenrichment culture had no beneficial effect on the growth of any of the *Salmonella* strains after 6 and 8 h. After 6 h of incubation, the average number of salmonellae was 9.6×10^3 CFU/ml in the samples not shaken and 2.6×10^3 CFU/ml in the samples shaken ($P = 0.2$). The average number of salmonellae after 8 h was 2.3×10^5 CFU/ml in the samples not shaken and 9.7×10^4 CFU/ml in the samples shaken ($P = 0.4$). However, after incubation for 24 h, shaking increased the number of CFU per milliliter by 1 log unit ($P = 0.0003$), as the average number reached 6.5×10^9 CFU/ml, compared to 5.8×10^8 CFU/ml for those not shaken.

Analysis of the results obtained by BHI, TSB, and BPW showed no significant difference in the abilities of the three different preenrichment media to support growth of *Salmonella* after 6 and 8 h. After 6 h, the average number of salmonellae reached 9.6×10^3 CFU/ml in BPW, 1.2×10^3 CFU/ml in TSB, and 1.4×10^3 CFU/ml in BHI ($P = 0.15$). After 8 h, these values were 2.3×10^5 CFU/ml in BPW, 6.4×10^4 CFU/ml in TSB, and 4.3×10^4 CFU/ml in BHI ($P = 0.21$). After incubation for 24 h, both of the nutrient-rich media, BHI and TSB, resulted in increased growth of *Salmonella* (BPW, $\sim 5.8 \times 10^8$ CFU/ml; TSB, $\sim 6.1 \times 10^9$ CFU/ml; BHI, $\sim 5.3 \times 10^9$ CFU/ml [averages]; $P = 0.005$). These average results were also reflected on the strain level. The PCR results obtained with the different preenrichment media indicated no difference in the inhibition of PCR among the three media. After 8 h, the average C_T values obtained were 20 for BPW, 21.5 for TSB, and 21.1 for BHI. After 24 h of preenrichment, these values were 15.3 for BPW, 16.7 for TSB, and 15.2 for BHI.

No difference in growth was observed for any of the strains tested when sodium pyruvate or egg yolk was added, regardless of the concentration (data not shown).

Addition of tergitol 4 and sulfamandelate to preenrichment

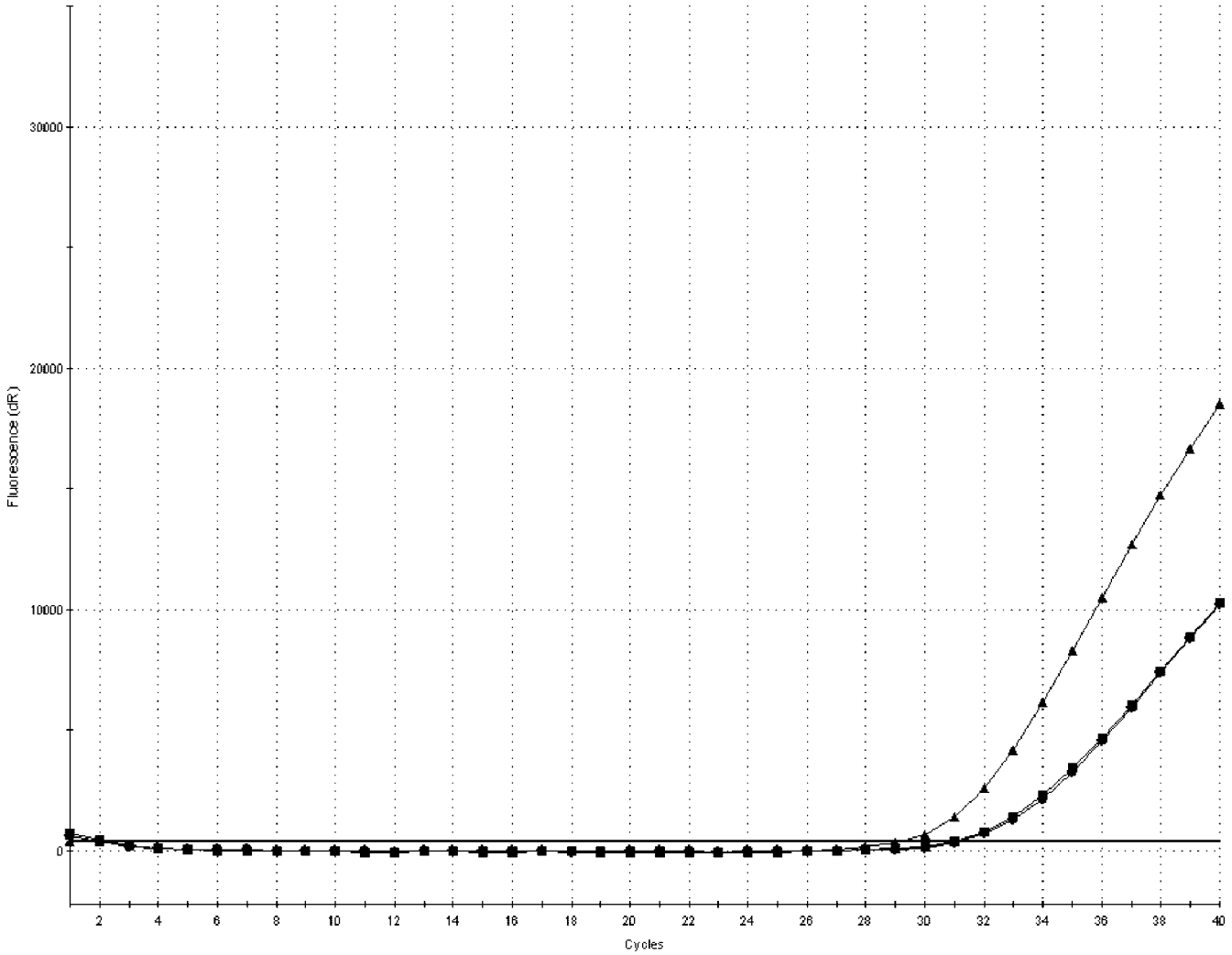

FIG. 1. Amplification plot (FAM) showing the difference in amplification curves obtained by sampling 1 ml (●), 2 ml (■), and 5 ml (▲) of minced pork meat inoculated with 1 to 10 CFU of *S. enterica* serovar Livingstone and enriched for 8 h at 37°C. Each amplification curve represents an average of four replicates.

of minced pork meat had no effect on the C_T values, regardless of the concentrations applied. Malachite green oxalate salt and deoxycholate resulted in slightly higher C_T values after 20 h of preenrichment, while no differences in C_T values were observed after 6 and 8 h. Addition of novobiocin (20 and 50 mg/liter) and brilliant green (10 mg/liter) had a tendency to improve the PCR results obtained after 8 h of preenrichment.

Optimization of sample preparation. Increasing the sampling volume of BPW to 5 ml resulted in an improved detection limit and steeper amplification curves, as shown in Fig. 1. The average C_T values were 31.8 for a 1-ml volume, 31.6 for a 2-ml volume, and 29.7 for a 5-ml volume. Washing of pellets produced higher C_T values and flatter PCR amplification curves. Some samples were not even PCR positive following the two washing steps.

Loss of DNA during extraction. The amount of DNA lost in the extraction procedure when extracting purified DNA resulted in an average increase in C_T values of 4.8 (Table 1). The DNA losses were more pronounced with the higher concentrations of DNA.

Table 2 shows the reverse correlation between the amount of paramagnetic particles and the C_T values. The lowest C_T values were obtained with 90 μl of paramagnetic particles. The effect of increasing the amount of paramagnetic particles was not pronounced, and the C_T value was lowered approximately 1, on average, when using 90 μl of paramagnetic particles compared to 60 μl. Steeper amplification curves were, however, obtained with 90 μl of paramagnetic particles, as shown in Fig. 2.

Increasing the concentration of DNA. Ambiguous results were obtained when attempting to increase the concentration of DNA by eluting in reduced volumes. Elution of DNA in a 25-μl volume resulted in very high and nonreproducible C_T values. Reducing the elution volume from 100 to 50 μl did not improve the results.

Increasing the PCR template DNA. As shown in Fig. 3, increasing the amount of template DNA markedly reduced the

TABLE 2. C_T values obtained in PCR comparing duplicate samples with similar DNA concentrations extracted with 60, 75, or 90 μl of paramagnetic particles per sample

DNA concn (ng/μl)	Vol of paramagnetic particles (μl/sample)	C_T value			
		Salmonella/FAM		IAC/HEX	
0.1	60	22.1	22.2	27.0	27.0
0.1	60	22.2	22.3	26.9	26.8
0.1	60	22.7	22.8	27.1	26.6
0.1	75	21.8	21.9	26.9	26.8
0.1	75	22.7	22.5	26.6	26.5
0.1	75	21.9	21.9	26.9	26.6
0.1	90	21.9	21.6	26.9	26.7
0.1	90	21.8	21.9	26.6	26.3
0.1	90	21.8	21.8	26.5	26.4
0.05	60	24.5	24.6	26.6	27.1
0.05	60	23.8	23.7	26.8	27.1
0.05	60	24.1	24.3	26.5	26.9
0.05	75	23.6	23.5	27.3	26.6
0.05	75	23.1	23.1	26.7	26.9
0.05	75	24.5	24.4	26.8	26.5
0.05	90	23.9	23.9	26.7	26.8
0.05	90	23.3	23.4	26.8	27.2
0.05	90	23.2	23.4	27.2	26.5
0.01	60	26.9	26.9	27.1	26.8
0.01	60	26.7	26.3	26.4	26.6
0.01	60	26.9	27.6	27.0	27.4
0.01	75	24.8	24.9	26.8	26.7
0.01	75	26.3	26.8	26.8	27.2
0.01	75	25.0	24.9	26.8	27.0
0.01	90	24.8	25.2	26.5	26.7
0.01	90	25.7	25.7	27.1	26.5
0.01	90	24.7	24.9	26.9	27.2
0.005	60	26.7	26.6	26.7	26.7
0.005	60	27.2	27.4	27.0	26.8
0.005	60	27.3	27.3	26.9	27.1
0.005	75	26.6	26.6	27.0	26.5
0.005	75	26.0	26.3	26.7	26.8
0.005	75	27.8	27.6	27.1	27.2
0.005	90	26.2	26.2	27.4	26.8
0.005	90	25.8	25.6	26.9	26.8
0.005	90	26.3	26.3	27.0	26.8

C_T values and resulted in steeper amplification curves. There was a reverse correlation between the amount of template DNA and the C_T value, and the lowest C_T values were obtained by addition of 20 μl of template DNA. Addition of 10 μl, compared to 5 μl, of template DNA also reduced the C_T values. The average C_T value was reduced from 30.2 for 5 μl of template DNA to 29.1 for 10 μl of template DNA and to 28.6 for 20 μl of template DNA for the samples inoculated with 1 to 10 CFU and from 28.6 for 5 μl of template DNA to 27.1 for 10 μl of template DNA and to 26.6 for 20 μl of template DNA. However, the most significant effect was the steepness and reproducibility of the curves.

Validation against a reference culture method. The reference culture method identified 33 *Salmonella*-positive and 17 *Salmonella*-negative minced pork meat samples. For the 12-h PCR method, these values were 32 and 18, respectively. The C_T values of the PCR-positive samples inoculated with 1 to 10 CFU were in the range of 23.3 to 37.8, with an average of 29.6 (Table 3). Samples inoculated with 10 to 100 CFU gave C_T values in the range of 21.1 to 26.7, with an average of 24.6 (Table 3). The relative AC of the PCR method was 98%, its relative sensitivity was 97%, and its relative specificity was 100%.

The reference culture method and the PCR method identified 29 *Salmonella*-positive and 21 *Salmonella*-negative poultry samples, resulting in a relative AC, sensitivity, and specificity of 100%. The C_T values of the PCR-positive samples were in the range of 23.8 to 33.2, with an average of 27.7 (Table 3).

For details of the PCR results obtained by addition of selective reagents to BPW or reducing the elution, volume, see the supplemental material.

DISCUSSION

The 12-h PCR described in the present study is intended as a diagnostic tool for routine use in the meat industry, and therefore a high degree of robustness and reproducibility is imperative. As a tool for ensuring food safety and thereby public health, the method has to be reliable and consistent, day after day, in the hands of different personnel and on different sample matrices. Preliminary studies revealed that the limited preenrichment period of 8 h in BPW was unable to produce sufficient cell counts to meet these demands; thus, crucial steps of the method were optimized to improve sensitivity and robustness.

The concept of proving the presence or absence of *Salmonella* with only 8 h of preenrichment entails the need for optimization of this growth phase to yield the greatest possible amount of cells. The majority of the existing culture methods recommend BPW for resuscitation and preenrichment of *Salmonella* (2). The findings of the present study gave no reason to alter this. This point was further emphasized by the fact that shaking of the preenrichment cultures, and thereby improving the accessibility of the nutrients, did not increase the number of salmonellae during short-term incubation. This could be due to overgrowth of competitive flora, as found in other studies (33). Fricker (15) found BPW to be superior to lactose broth for preenrichment of *Salmonella* from sewage sludge. Other studies have shown no significant difference in the diagnostic sensitivity of BPW and universal preenrichment broth in an examination of fecal samples from swine (19). Similar results were found for BPW, TSB, and glucose mineral salt medium used for preenrichment of frozen or fresh samples of minced meat (35).

Adding growth-promoting reagents to BPW did not seem to provide *Salmonella* with a competitive edge, so as a last resort, addition of selective reagents directly to BPW was attempted. According to reference culture methods, a selective advantage is introduced when transferring preenriched cells to the next enrichment step (2, 4). However, because of the time limitations of the present method, this was not possible. In the present study, the experiments with selective reagents were performed with freeze-stressed *Salmonella*, taking into account the potential presence of damaged cells in the samples. During slaughter of pigs in Denmark, wind chilling is applied, rendering part of the bacterial flora freeze damaged. The lack of a beneficial effect of addition of most of the selective reagents could be due to the restriction of resuscitation and growth of the stressed *Salmonella* cells caused by the selective pressure in the preenrichment media. Delayed addition of selective reagents could be an alternative way to overcome this problem.

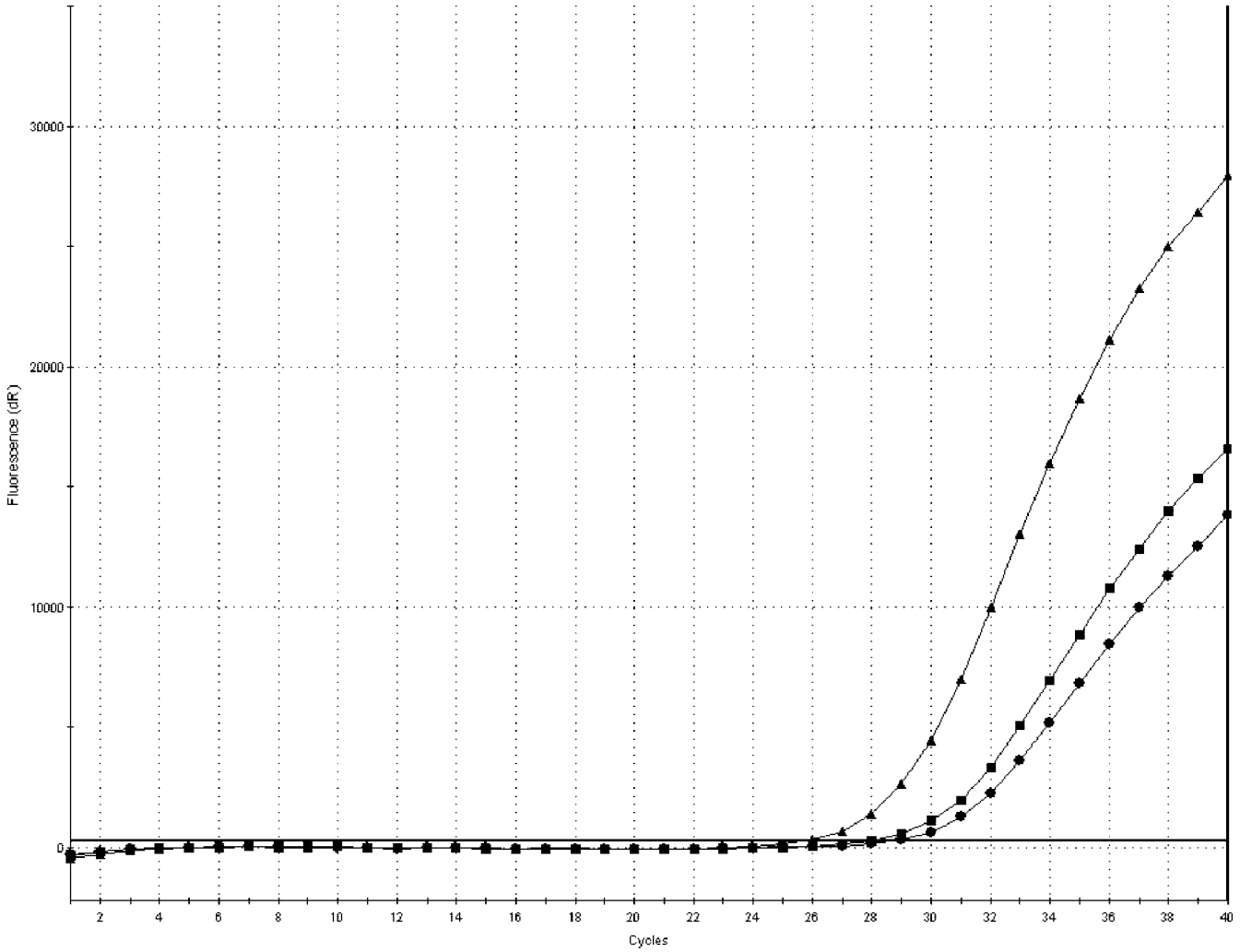

FIG. 2. Amplification plot (FAM) showing the difference in amplification curves obtained from a sample containing 0.01 ng/μl DNA extracted with 60 (●), 75 (■), or 90 (▲) μl of paramagnetic particles, respectively. The amplification curves represent average values of duplicate analyses.

Joosten et al. (21) showed increased recovery of *Salmonella* in infant formula containing high levels of probiotic microorganisms when malachite green was added to the preenrichment medium at a concentration of 100 mg/liter. However, the recovery rate improved when nonfat dry milk powder was added to the preenrichments to reduce the toxicity of malachite green toward *Salmonella*, which was also shown previously by van Schothorst and Renaud (36). In a study by Blivet et al. (9), malachite green (20 mg/liter) and brilliant green (5 mg/liter) were found to inhibit the growth of various *Salmonella* strains, while novobiocin (up to 40 mg/liter) enhanced their growth. Novobiocin addition (22 mg/liter) has likewise been reported to increase the recovery of *Salmonella* from fecal samples (20). Even though addition of novobiocin and brilliant green slightly improved the PCR results obtained after 8 h in the present study, it was decided not to add them to the BPW. The reference culture method does not include novobiocin or brilliant green, and adding these to the preenrichment would necessitate two preenrichment protocols of parallel samples, thus compromising the validation.

Another subject of importance is the PCR compatibility of the media used. In a study by Stone et al. (32), Rappaport-Vassiliadis and tetrathionate broths were found to be inhibitory to PCR, whereas BHI—as shown in this study—and selenite cystine broth were not. Eyigor et al. (13), on the other hand, found that a PCR performed directly on tetrathionate enrichment broth extracted by boiling was more sensitive than the reference culture method. Knutsson et al. (22) showed that both BPW and BHI inhibited the PCR. They also found that the r*Tth* PCR mixture, with the same DNA polymerase as in the present study, was less influenced by the presence of BPW than the Ampli*Taq* Gold PCR mixture. In other words, the DNA polymerase type plays a central role.

For the minced meat examined in the present study, a 5-ml volume resulted in markedly improved PCR results compared to a 1-ml volume. A high number of target organisms in a given sample can, however, influence the outcome of a larger sampling volume, and the possibility of overloading the PCR with target DNA should always be considered. Increasing the volume taken from preenrichment will, of course, result in higher

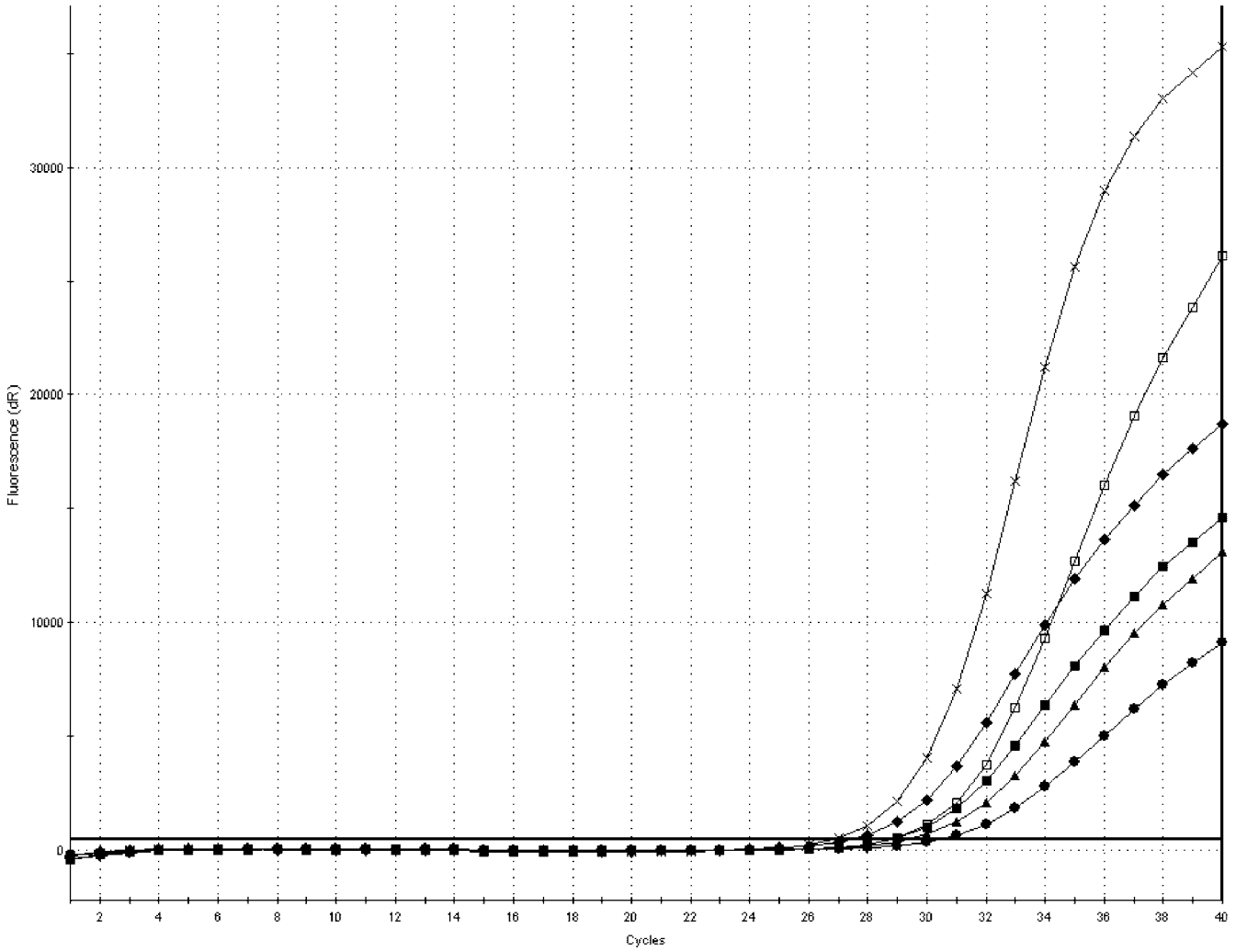

FIG. 3. Amplification plot (FAM) showing the difference in amplification curves obtained by analysis of 5, 10, and 20 μl of template DNA in the PCR from samples of minced pork meat inoculated with 1 to 10 or 10 to 100 CFU of *S. enterica* serovar Typhimurium CCUG 31939 and enriched for 8 h at 37°C. The amplification curves represent average values from triplicate analyses. ●, 1 to 10 CFU and 5 μl of template DNA; ■, 10 to 100 CFU and 5 μl of template DNA; ▲, 1 to 10 CFU and 10 μl of template DNA; ◆, 10 to 100 CFU and 10 μl of template DNA; □, 1 to 10 CFU and 20 μl of template DNA; ×, 10 to 100 CFU and 20 μl of template DNA.

numbers of target cells but, inconveniently, also increase the amount of other bacteria and PCR inhibitors. Accordingly, it is essential to find a balance where the advantage of the larger volume is not obscured by more inhibitors.

The automated DNA extraction procedure applied in this study was selected because the 12-h PCR method was developed for use in a routine laboratory with a high throughput and the need for a high degree of quality control. However, the DNA loss during extraction was shown to be high, as an average of 4.8 C_T units was lost by running pure DNA through the magnetic DNA extraction system. As a rule of thumb, 3.3 C_T units corresponds to 1 log unit. For example, for viral DNA in various clinical samples, Schuurman et al. (31) showed a DNA recovery of approximately 50% from magnetic DNA extraction. Ferreira-Gonzalez et al. (14) showed close to 100% recovery of viral DNA from plasma with a silicone-based kit. Previous in-house comparisons have shown automated DNA extraction with a KingFisher to be similar to, or better than, various manual and kit-based extraction methods (data not shown).

Increasing the amount of initial DNA in a PCR may result in rapid accumulation of high numbers of PCR products and in lower C_T values. However, when setting up a routine PCR test, the financial aspect should also be considered. Running a PCR with 20 μl of template DNA in a total volume of 50 μl of master mix would double the cost of PCR analysis compared to using 10 μl of template DNA in a total of 25 μl of master mix.

In conclusion, it was successfully demonstrated that the optimized 12-h PCR method for *Salmonella* detection produced results comparable to those of the reference culture method with artificially inoculated pork meat and poultry samples. Further studies with naturally contaminated samples are needed. The main advantage of the method developed is the reduced time of analysis, enabling faster release of *Salmonella*-free fresh meat. Moreover, the sample price and workload are

TABLE 3. Results obtained by analyzing artificially inoculated minced pork meat and poultry samples for the presence of *Salmonella* by the PCR method[a]

Sample	No. of CFU inoculated (organism)[b]	PCR (C_T value)	
		FAM[c]	HEX[d]
Minced pork meat			
1	1–10 (st)	28.7	33.3
2	1–10 (st)	37.7	32.9
3	1–10 (st)	NA[e]	32.6
4	1–10 (st)	36.4	32.4
5	1–10 (st)	33.7	32.1
6	1–10 (st)	33.6	32.3
7	1–10 (st)	37.8	32.4
8	1–10 (st)	33.7	32.5
9	1–10 (st)	35.2	32.1
10	1–10 (sl)	24.8	34.9
11	1–10 (sl)	23.7	34.8
12	1–10 (sl)	23.3	34.5
13	1–10 (sl)	25.5	33.5
14	1–10 (sl)	25.8	25.7
15	1–10 (sl)	24.5	34.1
16	1–10 (sl)	27.1	34.7
17	1–10 (sl)	29.9	33.9
18	1–10 (sl)	27.2	34.6
19	1–10 (sl)	24.7	34.9
20	10–100 (sl)	24.0	31.4
21	10–100 (sl)	24.2	32.1
22	10–100 (sl)	21.1	32.6
23	10–100 (sl)	23.6	31.5
24	10–100 (sl)	23.9	31.0
25	10–100 (sl)	25.6	31.2
26	10–100 (sl)	24.2	31.1
27	10–100 (sl)	25.1	31.5
28	10–100 (sl)	26.6	31.6
29	10–100 (sl)	22.1	32.0
30	10–100 (sl)	26.2	31.8
31	10–100 (sl)	25.5	31.3
32	10–100 (sl)	26.7	NA
33	10–100 (sl)	24.9	32.5
Poultry skin			
34	1–10 (se)	28.0	28.2
35	1–10 (se)	29.2	28.7
36	1–10 (se)	31.5	27.3
37	1–10 (se)	29.6	27.6
38	1–10 (se)	33.2	27.5
39	1–10 (se)	28.4	28.0
40	1–10 (se)	30.6	27.2
41	1–10 (se)	29.0	28.5
42	1–10 (se)	29.3	28.4
43	1–10 (se)	29.4	28.9
44	1–10 (se)	28.3	28.1
45	1–10 (se)	29.7	28.6
46	1–10 (se)	28.2	27.3
47	1–10 (se)	28.8	28.1
48	1–10 (se)	29.8	27.5
49	1–10 (st)	24.8	27.3
50	1–10 (st)	26.7	27.5
51	1–10 (st)	26.9	27.7
52	1–10 (st)	26.3	27.4
53	1–10 (st)	27.5	28.2
54	1–10 (st)	25.2	27.3
55	1–10 (st)	26.6	28.3
56	1–10 (st)	25.1	27.4
57	1–10 (st)	24.9	27.5
58	1–10 (st)	25.5	27.3
59	1–10 (st)	25.6	27.7
60	1–10 (st)	26.9	25.8
61	1–10 (st)	23.8	25.9
62	1–10 (st)	25.7	25.9

[a] All samples were found positive by the reference culture method.
[b] st, *S. enterica* serovar Typhimurium; sl, *S. enterica* serovar Livingstone; se, *S. enterica* serovar Enteritidis.
[c] *Salmonella* target.
[d] Internal amplification control.
[e] NA, no amplification.

markedly reduced compared to those of the reference culture method.

The strategies described in the present study are, in most cases, not unique to the detection of *Salmonella* but could be used to improve the sensitivity and/or shorten the preenrichment time of other real-time PCR-based methods.

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Research article

Validation of a same-day real-time PCR method for screening of meat and carcass swabs for *Salmonella*

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Abstract

Background: One of the major sources of human *Salmonella* infections is meat. Therefore, efficient and rapid monitoring of *Salmonella* in the meat production chain is necessary. Validation of alternative methods is needed to prove that the performance is equal to established methods. Very few of the published PCR methods for *Salmonella* have been validated in collaborative studies. This study describes a validation including comparative and collaborative trials, based on the recommendations from the Nordic organization for validation of alternative microbiological methods (NordVal) of a same-day, non-commercial real-time PCR method for detection of *Salmonella* in meat and carcass swabs.

Results: The comparative trial was performed against a reference method (NMKL-71:5, 1999) using artificially and naturally contaminated samples (60 minced veal and pork meat samples, 60 poultry neck-skins, and 120 pig carcass swabs). The relative accuracy was 99%, relative detection level 100%, relative sensitivity 103% and relative specificity 100%. The collaborative trial included six laboratories testing minced meat, poultry neck-skins, and carcass swabs as un-inoculated samples and samples artificially contaminated with 1–10 CFU/25 g, and 10–100 CFU/25 g. Valid results were obtained from five of the laboratories and used for the statistical analysis. Apart from one of the non-inoculated samples being false positive with PCR for one of the laboratories, no false positive or false negative results were reported. Partly based on results obtained in this study, the method has obtained NordVal approval for analysis of *Salmonella* in meat and carcass swabs. The PCR method was transferred to a production laboratory and the performance was compared with the BAX *Salmonella* test on 39 pork samples artificially contaminated with *Salmonella*. There was no significant difference in the results obtained by the two methods.

Conclusion: The real-time PCR method for detection of *Salmonella* in meat and carcass swabs was validated in comparative and collaborative trials according to NordVal recommendations. The PCR method was found to perform well. The test is currently being implemented for screening of several hundred thousand samples per year at a number of major Danish slaughterhouses to shorten the post-slaughter storage time and facilitate the swift export of fresh meat.

Background

One of the major sources of human *Salmonella* infection is meat, including pork and poultry [1,2] and therefore efficient and rapid monitoring of *Salmonella* in the meat production chain is necessary. Traditional bacteriological detection of *Salmonella* in foods and environmental samples is costly, laborious, and time-consuming, requiring 3–7 days to obtain a confirmed result [3]. Thus, rapid and cost-effective detection of *Salmonella* is of major interest to the food industry and the public. Real-time PCR technology offers several advantages compared with classical bacteriology in terms of speed, detection limit, potential for automation, and cost [4]. However, it is essential that new PCR methods are reliable, robust and comply with the legislative demand of detecting as few as one *Salmonella* bacterium per 25-g sample. Furthermore, they should be validated against reference culture methods, and last, but not least, be sufficiently robust to be transferred from the expert laboratory to end users.

There are several real-time PCR methods available for the detection of *Salmonella* in various kinds of food [5,6] and carcass swabs [7]. Furthermore, a number of commercial real-time PCR systems have been validated for testing of *Salmonella* in meat and swab samples [5,8-10]. Some of these systems detect *Salmonella* as fast as 9–10 h in meat samples (iQ Check Salmonella II, Bio-Rad, Hercules, CA and GeneDisc, GeneSystems, Bruz, France), but the total time for analysis of carcass swab samples is 17–20 h. Recently, a non-commercial real-time PCR method for detection of *Salmonella* in milk powder [11] has been validated in a multicenter trial. However, to our knowledge, there are no reports on multicenter validation trials where non-commercial methods are evaluated for the detection of *Salmonella* in meat or carcass swabs using real-time PCR.

The objective of this study was to validate a previously developed real-time PCR method [6,12,13] for use as a routine and on-site analysis method for the meat industry. The validation study was performed according to the protocol recommended by the validation body of the Nordic countries (NordVal) [14,15], including comparative and collaborative trials on minced pork and veal meat, chicken neck-skins and pig carcass swab samples. The method is based on a shortened (compared to the NMKL-71 method) pre-enrichment in buffered peptone water (BPW) followed by automated DNA purification and subsequent detection using real-time PCR. In this method, a part of the *ttr*RSBCA locus specific for *Salmonella* is amplified giving a high selectivity [6]. The PCR method used includes an internal amplification control (IAC), making it useful as a diagnostic tool. The overall time for the analysis of meat samples is 14 h, and for carcass swab samples 16 h. Both time-spans are operational for two-shift work at slaughterhouses. The method has on the basis of results obtained in this study together with already published data on selectivity [6] gained NordVal approval and is currently being implemented at major Danish meat producers.

Results

Comparative trial

The comparative trial was conducted in accordance with the guidelines provided by NordVal [15] and included the matrices meat (minced pork and veal meat as well as poultry neck-skins) and environmental samples (swabs from pig carcasses). The relative detection level, accuracy, sensitivity and specificity were evaluated for the real-time PCR method in comparison with the reference culture-based method currently in use (Table 1)[3].

The detection level of the two methods was 1–10 CFU/25 g sample (corresponding to a relative detection level of 100%) in all cases except for the swabs inoculated with *S*. Enteritidis, where it was 10–100 CFU/25 g for the NMKL method (relative detection level > 100%) (data not

Table 1: Results obtained in the comparative trial by the real-time PCR and the reference culture method [a, b].

Sample type[c]	No. of samples						% Value[d]			κ[e]
	N	PA	NA	FN	TP	FP	AC	SE	SP	
Minced meat	60	30	30	0	0	0	100	100	100	1.00
Poultry neck-skins	60	27	31	0	2	0	97	107	100	0.97
Pig carcass swabs	120	21	98	1	0	0	99	95	100	0.97
TOTAL	**240**	**78**	**159**	**1**	**2**	**0**	**99**	**103**	**100**	**0.97**

[a] PA: Positive Agreement, NA: Negative Agreement, TP: True Positive, FN: False Negative, FP: False Positive, AC: Relative Accuracy, SE: Relative Sensitivity, SP: Relative Specificity, N = PA +NA + FN + TP + FP.
[b] Results are given after confirmation.
[c] Matrices as defined by NordVal [15]; matrix meat: minced meat (raw pork and veal) and poultry neck skins, matrix environmental samples: pig carcass swabs. Meat samples were artificially contaminated and swab samples potentially naturally contaminated.
[d] See Materials and Methods for accuracy, sensitivity and specificity equations.
[e] Cohen's kappa calculated according to NMKL procedure no. 20 [26].

shown). To determine the relative accuracy, sensitivity and specificity, a total of 240 samples representing meat and environmental samples were analyzed by the PCR and NMKL methods (Table 1). A total of 80 out of 240 samples gave positive results by real-time PCR, compared with a total of 79 by the culture-based method. Two samples showed positive deviation (true positives by the PCR method) and one negative deviation (false negative by the PCR method) (Table 1). A very good agreement between the two methods was obtained using Cohen's kappa (Table 1).

Collaborative trial

The purpose of the collaborative trial was to determine the variability in the results obtained by the real-time PCR method detecting *Salmonella* in identical samples. The trial was conducted in accordance with the guidelines provided by NordVal [15]. The samples and the other contents of the ring trial kit sent out to the participants were found to be stable during the period of the trial (data not shown). The influence of the refrigerated transit was investigated prior to the collaborative trial, and no detrimental effects were found after three days (data not shown).

Six laboratories participated in the collaborative trial, and valid results were obtained from five of the laboratories and used for the statistical analysis (Table 2). In agreement with the predefined criteria, results from one participant were excluded due to failure in the PCR analysis (lack of amplification in the positive control and several samples with no amplification of either the target or the IAC). The unexpected PCR results obtained by this participant were probably caused by a delay in the transport of the ring trial package (> 5 days). Statistical analysis of the results from the remaining five laboratories gave a relative specificity, sensitivity and accuracy of 100% for all of the tested matrices at all three inoculation levels, except for the relative accuracy for swab samples which was 83% when all inoculation levels were analyzed together. For the positive control samples containing *Salmonella* DNA, a Ct value of 32.6 ± 1.6 was obtained for the five laboratories. There were small variations in the Ct values obtained for duplicate samples of the same matrix at the same spiking level analyzed at each laboratory (standard deviation 0.0–2.7) as well as for the same sample analyzed by each laboratory (standard deviation 1.1–1.9).

External validation

In order to evaluate the performance of the real-time PCR method on-site, it was transferred and implemented at a production laboratory previously using PCR-based analysis with the BAX system. Artificially contaminated pork filet samples (n = 39) were analyzed in parallel with the real-time PCR and BAX methods. In general, a good agreement (κ = 0.77) was found between the two methods

Table 2: Collaborative trial: PCR results for *Salmonella* in artificially contaminated meat samples and pig carcass swabs.

Sample type	Participant no.	Ct values for replicates from indicated level of spiking (CFU/25 g)[a]		
		0	1–10	10–100
Carcass swabs	1	> 36, > 36	17, 19	19, 19
	2	> 36, > 36	14, 16	16, 16
	3	> 36, > 36	15, 17	16, 16
	4	> 36, > 36	16, 18	17, 17
	5	> 36, 34	16, 18	19, 17
	Mean ± SD[b]	n.a.[c]	16.5 ± 1.3	17.1 ± 1.3
Poultry neck-skins	1	> 36, > 36	28, 28	25, 24
	2	> 36, > 36	26, 26	24, 24
	3	> 36, > 36	29, 28	25, 24
	4	> 36, > 36	24, 25	23, 22
	5	> 36, > 36	25, 25	22, 23
	Mean ± SD[b]	n.a.	26.6 ± 1.8	23.6 ± 1.1
Minced meat	1	> 36, > 36	20, 21	17, 17
	2	> 36, > 36	21, 20	16, 18
	3	> 36, > 36	19, 19	16, 15
	4	> 36, > 36	18, 18	13, 14
	5	> 36, > 36	18, 18	17, 13
	Mean ± SD[b]	n.a.	19.4 ± 1.9	15.4 ± 1.8

[a] Ct values below 36 were considered as positive responses.
[b] The mean and standard deviation calculated for all the replicate analysis of the same sample independent of the participant.
[c] n.a.: not applicable

based on the results from the 39 artificially contaminated samples (Tables 3 &4). The real-time PCR method detected 33 of the 39 samples inoculated with *Salmonella*, whereas the BAX system detected 34 of the 39 samples.

Discussion

The real-time PCR method validated in the present study is intended as a diagnostic tool for routine use in the meat industry, and therefore has specific demands on speed, ease of automation as well as robustness and reproducibility. Furthermore, the method must be specific for *Salmonella* and have detection limit comparable with or better than the culture-based methods in use today as official methods. Using the PCR method, the total time for the analysis of *Salmonella* in meat samples was decreased from at least 3 days for the standard culture-based method [3] to 14 h for meat samples and 16 h for swabs. The time for analysis is comparable with the fastest validated DNA-based analysis kit (e.g. from Bio-Rad and GeneSystems) on the market for meat samples and 1–3 h shorter for swab samples. For the meat producer, this means that the meat can be released faster, leading to decreased costs for storage and prolonged shelf life at the retailers. Implementing this method would allow faster release of *Salmonella*-free fresh meat and meat products.

The sample preparation in the PCR method consists of non-selective enrichment in BPW followed by centrifugation and automated DNA extraction. The use of automated DNA extraction in combination with the closed system of real-time PCR provides a fast and less laborious method with minimized risk of contamination. Furthermore, the real-time PCR method can easily be adapted to include the dUTP-uracil-*N*-glycosylase (UNG) system, minimizing the risk of carryover contamination [16]. The PCR reagents used in the method can be mixed in advance, distributed in smaller, ready-to-use quantities, and frozen at -20°C for up to 3 months [17]. These features are a major benefit for on-site use of the test at the slaughterhouses. The method is an open-formula technique, i.e., the reagents and target gene, etc., are known, in contrast to commercial kits. However, further decreasing the total time for analysis to below 8 h will certainly be even more beneficial to industry and is a challenge in the further developing of the method.

The prevalence of *Salmonella* in Danish pork meat and broiler flocks is low (0.9% and 2.2%, respectively [18]). Therefore, samples artificially contaminated with *Salmonella* in the exponential growth phase stressed by a cold storage overnight to simulate the condition under production of poultry and pork meat were used for the majority of the samples included in the validation study. This alternative to naturally contaminated samples is in compliance with international guidelines [15,19]. However, naturally contaminated swab samples were used for the comparative trial. The NMKL-71 (1999) method [3] was chosen as the reference method because it is used in the Nordic countries instead of the ISO 6579:2002 method [20]. The difference in the two methods is that in the NMKL method only one selective enrichment media is used Rappaport Vassiliades soy broth (RVS) instead of two in the ISO method (RVS and Muller-Kauffmann Tetrathionate-Novobiocin broth, MKTTn). The methods have been determined to be equal to the respective part of the ISO method [21].

The real-time PCR method amplifies a part of the *ttrRSBCA* locus used for tetrathionate respiration in *Salmonella*. The relative selectivity of the PCR assay (primers and probes) has previously been found to be 100% when tested on 110 *Salmonella* strains and 87 non-*Salmonella* strains [6]. Therefore, this parameter was excluded from the comparative test performed in this study, in accordance with NordVal guidelines. The relative accuracy, sensitivity and specificity were evaluated for the PCR method in comparison with the standard culture-based method currently in use for detection of *Salmonella* [3] according to the NordVal protocol

Table 3: Results obtained by the real-time PCR and the *Salmonella* BAX PCR in the external validation.

***Salmonella* level (CFU/25-g sample)**	**No. of samples analyzed**	**Result obtained by the PCR and BAX methods[a]**				
		PA	PD	ND	NA	Inconc./+
1000	3	3	0	0	0	0
100	3	3	0	0	0	0
10	9	7	0	0	2	0
5	12	10	1	0	0	1
2	12	9	0	1	2	0
TOTAL	**39**	**32**	**1**	**1**	**4**	**1**

[a] PA: positive by PCR and BAX methods, PD: positive by PCR and negative by BAX, ND: negative by PCR and positive by BAX, NA: negative by PCR and BAX methods, inconc./+: inconclusive result by PCR (need re-analysis) and positive by BAX.

Table 4: Detailed results from the external validation study.

Salmonella serotype	Inoculation level (cfu/25 g)	Real-time PCR[a]			Salmonella BAX Detection System
		Ct-value for Salmonella	Ct-value for IAC	Final result	Final result
Infantis	1000	20.05	27.89	Positive	Positive
	100	21.66	29.09	Positive	Positive
	10	27.14	28.68	Positive	Positive
	10	30.59	28.95	Positive	Positive
	10	24.92	28.89	Positive	Positive
	5	29.42	29.09	Positive	Positive
	5	26.57	28.81	Positive	Positive
	5	26.29	27.66	Positive	Positive
	5	26.63	28.79	Positive	Positive
	2	27.70	28.42	Positive	Positive
	2	25.68	28.08	Positive	Positive
	2	27.86	28.56	Positive	Positive
	2	27.20	28.90	Positive	Positive
Agona	1000	22.47	28.97	Positive	Positive
	100	24.70	27.93	Positive	Positive
	10	**> 36**	**29.21**	**Negative**	**Negative**
	10	**> 36**	**29.07**	**Negative**	**Negative**
	10	26.04	28.93	Positive	Positive
	5	28.47	28.76	Positive	Positive
	5	**32.93**	**28.53**	**Positive**	**Negative**
	5	29.84	28.92	Positive	Positive
	5	32.17	27.90	Positive	Positive
	2	**> 36**	**28.76**	**Negative**	**Positive**
	2	**> 36**	**29.07**	**Negative**	**Negative**
	2	33.22	28.77	Positive	Positive
	2	30.61	27.96	Positive	Positive
Infantis	1000	19.59	29.01	Positive	Positive
	100	23.74	28.86	Positive	Positive
	10	25.55	28.45	Positive	Positive
	10	24.85	28.40	Positive	Positive
	10	26.82	28.36	Positive	Positive
	5	29.82	29.10	Positive	Positive
	5	29.03	28.16	Positive	Positive
	5	24.77	28.28	Positive	Positive
	5	**> 36**	**> 40**	**Inconclusive**	**Positive**
	2	28.61	27.88	Positive	Positive
	2	26.24	28.79	Positive	Positive
	2	26.02	28.82	Positive	Positive
	2	**> 36**	**28.63**	**Negative**	**Negative**

Results from 39 pork meat samples inoculated with salmonella at different levels and analyzed in parallel on-site using the real-time PCR and the Salmonella BAX methods.

[a] Samples with a Ct value > 36 is considered negative if the Ct value for the IAC is < 40 and inconclusive if a Ct > 40 is obtained for the IAC. According to the Method Directive for the PCR method, re-analysis of the extracted DNA by PCR is then needed.

(Table 1). Two of the artificially contaminated poultry neck-skins were found positive by the real-time PCR method and negative by the reference method. These samples were considered as true positives because according to ISO 20838:2006 [22] no further verification of positive samples is necessary, as the real time PCR analysis contains a DNA probe specific for the target *Salmonella* gene (*ttrRS-BCA* locus). The relative sensitivity for the matrices meat and environmental samples, as well as when all the samples were analyzed together were above 95%, which is the limit considered acceptable according to NordVal [15]. No recommendations concerning the levels for the relative accuracy and relative specificity are given in either the guideline [15] or in the ISO16140 standard [19].

In the collaborative study, complete agreement between the real-time PCR method and the culture-based reference method was obtained for all test characteristics for minced pork and veal meat as well as for poultry neck-skin samples. For carcass swabs, one of the samples that were not artificially contaminated was positive when analyzed by one of the laboratories. However, investigations after the finalization of the trial pointed to a mix-up of two samples during the set-up of the PCR plate, which presents a reasonable explanation for this false-positive result. One of the participants was excluded from the study, due to too long transportation time (> 5 days) which has a detrimental effect on the PCR master mix. There are some limitations to this study that should be taken into consideration when implementing the method at other laboratories. Firstly, only one brand of PCR thermo cycler was used in the study. It has previously been reported that PCR results might vary considerable between different thermocyclers [12] and it might be necessary to adjust reagent concentrations and the temperature program slightly to optimize the method. Secondly, the enrichment step of the method was only performed at the expert laboratory and pellets were sent out for DNA extraction and PCR analysis. Thus the reproducibility was assessed for the DNA extraction and PCR steps. This procedure was approved in advance by NordVal. The participating laboratories were experienced laboratories that were familiar with culture based methodologies. However, in other guidelines for collaborative studies, such as ISO 16140, it is recommended that the complete procedure is performed by all participating laboratories [19].

In the last part of the study, the robustness of the method was verified externally for artificially contaminated pork samples. No significant difference in the result for the real-time PCR method and a commercial SYBR-Green PCR-based analysis system (BAX) was found. However, results were available after 14 h for the real-time PCR method, compared with 20–24 h for the BAX system. In this study, two samples inoculated with a very low level (estimated 2 CFU/25 g) and two samples inoculated at 10 CFU/25 g were negative in both methods, most likely indicating that no surviving *Salmonella* actually were present in the sample. Freezing at -18 °C will kill some of the inoculated *Salmonella* cells, thereby affecting the possibility for further detection using BAX or the real-time PCR method. Furthermore, the BAX system failed to detect one sample inoculated with 5 CFU/25 g of *S*. Agona. The same sample was detected using the real-time PCR method although the Ct value was rather high (Ct value of 33). Finally, two samples (5 CFU/25 g of *S*. Infantis and 2 CFU/25 g of *S*. Agona) were not detected by the real-time PCR method although being positive with the BAX system. For one of these samples, however, the IAC was negative as well, prompting a re-examination of the sample. However, at low inoculation levels the cell number added can vary due of statistical reasons thereby affecting the probability of detection [23]. From these data, it can be concluded that the real-time PCR is equivalent to the BAX system in detecting *Salmonella* in artificially contaminated meat samples

Conclusion

In conclusion, the real-time PCR method was validated in comparative and collaborative trials according to guidelines given by NordVal. The PCR method was found to perform well. Results from this study together with published data on selectivity of the real-time PCR assay [6] formed the basis for obtaining NordVal approval as an alternative method for detection of *Salmonella* in meat and environmental (carcass swabs) samples [24]. After a successful comparison with a commercially available SYBR-Green PCR-based method currently used by a number of meat producers, the real-time PCR method is now being implemented as a routine analysis method by leading poultry and pork producers in Denmark for qualitative detection of *Salmonella* in raw meat and carcass swabs.

Methods

DNA extraction

Five-ml aliquots from the pre-enrichments were drawn for DNA-extraction. For the automated DNA extraction method, the aliquots were centrifuged at 3000 × g for 5 min, and DNA-extraction performed on a KingFisher (Thermo Labsystems, Helsinki, Finland), as previously described [13], using a DNA isolation kit for blood, stool, cells and tissue (Magnesil KF, Genomic system, Promega, Madison, WI) as specified by the manufacturer with a total of 75 μl of magnetic particles.

Real-time PCR

A TaqMan real-time PCR method [6], targeting a region within the *ttrRSBCA* locus, for the specific detection of *Salmonella*, was employed as previously described [13] using 9 μl of the purified DNA as template in a total reaction volume of 25 μl.

Reference culture based method

The detection of *Salmonella* spp. was conducted in accordance with the recommendations from the Nordic Committee on Food Analyses (NMKL) [3] as previously described [13]. However, 25 g of sample (meat) or one swab was transferred to pre-heated buffered peptone water (1:10, BPW; Oxoid, Basingstoke, United Kingdom) and incubated at 37 °C for 18 ± 2 h.

Preparation of inoculum

To prepare the culture used for artificial inoculation in the comparative and collaborative trials, the *Salmonella*

strains were grown overnight at 37°C on 5% blood agar (BA) plates (Statens Serum Institute, Copenhagen, Denmark). One colony of each of the strains was transferred to 4 ml of Nutrient broth with NaCl (8.5 g/l NaCl and 20 g/l Nutrient Broth (BD 234000, BD Denmark, Brøndby, Denmark)), vortexed and incubated at 37°C for 3–4 hours. After the incubation, a 10-fold dilution series in 0.9% NaCl solution was performed to determine the concentration of the *Salmonella* cells. From the dilution series, 0.1 ml from each tube was spread on two 5% BA plates. The tubes were stored at 2–5°C for 16 to 20 hours and the 5% BA plates were incubated for 16 to 20 hours at 37°C and the colonies were counted. The samples were subsequently inoculated from a tube in the dilution series with a known concentration of *Salmonella* cells. At the time of inoculation, 0.1 ml was spread onto each of two BA plates to estimate the actual inoculation level.

For the on-site validation, three different strains of *Salmonella* (two *S*. Infantis and one *S*. Agona) previously isolated from pork meat were grown in Brain Heart Infusion (Oxoid CM0225) at 37°C for 24 hours resulting in approximately 2×10^9 CFU/ml. The next day, the cultures were 10-fold diluted using 0.85% NaCl + 1% peptone.

Sample preparation

Minced veal and pork meat were purchased at local retailers. Pig carcass swabs and poultry neck-skins were obtained from local abattoirs. Carcass swabs were sampled according to ISO 17604 [25] in accordance with EU directive 2073/2005/EC [26] employing the non-destructive swab method with gauze swabs. The sites on the pig carcass that were swabbed included the ham, back, belly and jowl.

After being transported cooled to the laboratory, the samples were analyzed using the real-time PCR method (DNA extraction and TaqMan PCR, as described above) and the reference culture method. Briefly, *Salmonella*-free (verified by the NMKL-71 method) fresh meat (25 g) or swab sample (one swab) was transferred to 225 ml (for meat samples) or 1:10 (weight of sample:volume of buffer for swabs) of BPW (37°C). Different levels of *Salmonella* (see "Comparative trial" and "Collaborative trial" below) were thereafter added. All the samples were pre-heated to 37°C and homogenized by hand for 20 seconds. After pre-enrichment at 37°C (12 ± 2 h for minced meat and neck-skins and 14 ± 1.5 for swabs), 5 ml aliquots were drawn for DNA-extraction and real-time PCR analysis using 9 µl of the extracted DNA. The enrichment was thereafter continued up to 18 hours according to NMKL-71 [3] and further analyzed according to that protocol.

Comparative trial

The comparative trial was designed and conducted according to the recommendations from NordVal [15]. To evaluate the relative detection level, artificially inoculated samples were analyzed by NMKL-71 and the real-time PCR method as described above. For each of the matrices of minced meat, poultry neck-skins and pig carcass swabs, one sample of 25 g (for meat and neck-skins) and one swab was left un-inoculated; six were inoculated with 1–10 CFU/25 g and six with 10–100 CFU/25 g. Half of the samples at each inoculation level were inoculated with *S*. Enteritidis CCUG 32352 and the other half with *S*. Typhimurium CCUG 31969.

To evaluate the relative accuracy, relative specificity and relative sensitivity of the real-time PCR method, minced pork and veal meat (n = 60, artificially contaminated), poultry neck-skins (n = 60, artificially contaminated) and swabs from pig carcasses (n = 120, potentially naturally contaminated) were used, see Table 1. The samples were analyzed by NMKL-71 and the PCR method as described above. For the minced meat, 30 samples were left un-inoculated; 15 samples were inoculated with *S*. Livingstone (in-house bacteria culture collection) 1–10 CFU per 25 g and 15 samples were inoculated with *S*. Typhimurium CCUG 31969 1–10 CFU per 25 g. For the poultry neck-skins, 31 samples were left un-inoculated, 15 samples were inoculated with 1–10 CFU *S*. Enteritidis CCUG 32352 per 25 g and 14 samples were inoculated with 1–10 CFU *S*. Typhimurium CCUG 31969 per 25 g. The pig carcass swab samples consisted of 120 non-inoculated samples from a Danish abattoir.

Collaborative trial

A collaborative trial involving six laboratories was performed to evaluate the robustness and reproducibility of the real-time PCR method testing identical samples. Laboratories belonging to Danish meat producers as well as other laboratories with the equipment used were selected for inclusion in the study. The reason for not including a larger number of participants was that it was not possible to find more than six laboratories that had the equipment and were willing to take part. The collaborative trial was designed and conducted according to the recommendations from NordVal [15] and included minced meat, poultry neck-skins and pig carcass swabs. The participating laboratories received pellets from 18 coded 5-ml samples (six from each matrix, see Table 2).

The samples for the collaborative trial were prepared as described above ("Sample preparation"). To produce the pellets included in the shipment, the supernatant was discarded after the centrifugation step, and the pellet kept at -20°C until shipped on ice to the trial participants. The samples were duplicate samples un-inoculated and inoculated artificially contaminated in duplicate with *S*. Typhimurium CCUG 31369 at two levels (1–10 CFU/25 g and 10–100 CFU/25 g) before enrichment, making it possible to assess the usefulness of the method at various infection levels.

The *Salmonella* status of all samples was confirmed by the reference culture method NMKL-71 [3] prior to and after spiking. The stability of the samples was examined using the real-time PCR method immediately after preparation, prior to commencement of the collaborative trial, during the period of analysis, as well after the trial was finished to verify the continued detection of *Salmonella*. The possible detrimental effect of shipping time at ambient temperature on the real-time PCR results was investigated, by analyzing a ring trial package after storage at room temperature for three days (the maximum shipment time to the participants was two days).

The shipment included a positive DNA control (1 μg/ml *S*. Typhimurium CCUG 31369) and a negative DNA control (1 μg/ml *Escherichia coli* O157 (Sample ID 077, Institute for Reference Materials and Measurements, Geel, Belgium)), a ready-to-use PCR mixture with added IAC, reagents for the magnetically based DNA extraction and the consumables for the DNA extraction and PCR analysis. To minimize any inter-laboratory variability (not attributable to the method performance), all the reagents necessary were supplied by the expert laboratory. At the participating laboratories, DNA extraction and PCR analysis were performed as described above. Real-time PCR at the participating laboratories was performed on an Mx3000 or Mx4000 real-time PCR system (Stratagene, La Jolla, CA). Each participant received a detailed protocol describing the DNA extraction, real-time PCR setup, real-time PCR run, and data analysis as well as a reporting form to record the obtained PCR results to return to the expert laboratory. The participants were also asked to return a file containing the real-time PCR runs. The participating laboratories were asked to use the negative template control (NTC), the process blank (a *Salmonella*-negative sample processed throughout the entire protocol) and the negative control to assign the threshold.

External validation

Slices of pork filet were obtained from a local supermarket, and aseptically cut into pieces of 25 grams. Thirty-nine pieces of pork filet were inoculated by adding 0.5 ml of an appropriate dilution of *Salmonella* cells (see "Preparation of inoculum") onto the surface of the meat resulting in the following estimated inoculation levels for each of the three strains: one sample containing approximately 1000 CFU/25 g, one sample containing approximately 100 CFU/25 g, three samples containing approximately 10 CFU/25 g, four samples containing approximately 5 CFU/25 g and four samples containing approximately 2 CFU/25 g. After inoculation, the meat samples were placed in a stomacher bag and frozen at -18°C for 24 hours in order to induce a slight freezing stress to the *Salmonella*, resembling the stress during blast-cooling as used by the Danish abattoir.

All 39 samples were analyzed by the real-time PCR method and the BAX Salmonella Detection System (BAX, DuPont Qualicon, Oxoid) using the following protocol. The 25-g sample was thawed overnight at 4°C, 225 ml pre-warmed BPW (37°C, Oxoid) was added, and the samples were then incubated at 37°C. After 10 hours, a 5-ml aliquot was drawn for DNA extraction and subsequent real-time PCR analysis as described above. The remaining BPW was further incubated at 37°C for an additional 8 hours, and samples were thereafter treated according to the manufacturer's instructions.

Statistical data analysis

The comparative validation study included three test characteristics: relative accuracy (AC), sensitivity (SE), and specificity (SP) (1) (see Table 1), and these were calculated and defined as previously described [27]. False negative (FN) results were defined as samples giving a negative result with PCR and a positive result with the NMKL-71 method. True positive (TP) results were defined as samples with positive PCR results and negative NMKL-71 results when obtained for artificially contaminated samples. Cohen's kappa (κ) was calculated as described by NMKL to quantify the degree of agreement between the two methods [28] ($\kappa > 0.80$ means very good agreement between the methods). This method was also used to evaluate the agreement between the real-time PCR and the BAX method in the on-site validation study.

For the collaborative validation study, the test reports and the real-time PCR analyses from the participating laboratories were carefully evaluated on return to the expert laboratory, and the results were approved for inclusion in the statistical analysis, unless they fell into at least one of the following two categories: (i) obvious performance deviation from the protocol and (ii) failed PCR analysis as shown in the included controls. The results obtained in the collaborative trial were analyzed according to the recommendations from NordVal [15]. SP was calculated for the un-inoculated samples by the following equation: $SP = (1 - [FP/N-]) \times 100\%$, where N- refers to the total number of samples not inoculated with Salmonella. SE was calculated for each level of spiking by the following equation: $SE = (TP/N+) \times 100\%$, where N+ refers to the number of artificially contaminated samples. AC was calculated for all levels of spiking by the following equation: $AC = ([PA + NA + FP]/N) \times 100\%$, where N refers to the number of samples tested.

Authors' contributions

CL participated in the design of the study, performed part of the experimental work for the collaborative study, performed the statistical analysis and drafted the manuscript. MHJ and MK planned and performed the experimental work on the comparative study. FH planned and per-

formed the experimental work for the external validation. JH conceived the study, obtained funding, helped to draft and critically read the manuscript. All authors read and approved the final manuscript.

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Diagnostic PCR: Comparative sensitivity of four probe chemistries. Josefsen, M.H., C. Löfström, H.M. Sommer and J. Hoorfar.

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Short Communication

Diagnostic PCR: Comparative sensitivity of four probe chemistries

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ABSTRACT

Three probe chemistries: locked nucleic acid (LNA), minor groove binder (MGB) and Scorpion were compared with a TaqMan probe in a validated real-time PCR assay for detection of food-borne thermotolerant *Campylobacter*. The LNA probe produced significantly lower Ct-values and a higher proportion of positive PCR responses analyzing less than 150 DNA copies than the TaqMan probe. Choice of probe chemistry clearly has an impact on the sensitivity of PCR assays, and should be considered in an optimization strategy.

An increasing number of alternative probe chemistries for real-time PCR are being marketed, such as minor groove binder (MGB) [1], Molecular Beacon [2], Scorpion [3], locked nucleic acid (LNA) [4] and Light Upon eXtension (LUX) [5]. The alternative probe technologies are based on different chemistries and claim to have some advantages compared with the conventional TaqMan DNA probes. However, there is a lack of diagnostic comparative studies. We thus compared three of the probe chemistries, namely LNA, MGB and the Scorpion technology (uni- and bi-molecular) to a TaqMan probe used in routine testing (Table 1). The probes were designed by the manufacturers to comply with the existing TaqMan probe and primers for an already validated real-time PCR assay for detection of food-borne thermotolerant *Campylobacter* (*Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari*) [6].

A prerequisite for successful probe-based real-time PCR is a strong binding of the probe during annealing. This is obtained by designing probes with a 5–10 °C higher melting temperature (T_m) than the primers. For TaqMan probes, this often results in 25–30 nucleotide long probes, which, in the case of a short specific region, can be difficult to design.

LNA probes have certain nucleic bases substituted by LNA monomers, i.e. nucleic acid analogs containing a 2′-O,4′-C methylene bridge, restricting the flexibility of the ribofuranose ring and rendering the monomer in a rigid bicyclic formation. This enhances the hybridization performance of LNA containing probes compared to classical TaqMan probes, and allows shorter probe designs. The incorporation of LNA monomers will increase the thermal stability of a duplex complementary DNA significantly (up to 8 °C per substitution) [7].

MGB probes have a moiety attached to the 3′-end of the probe, which folds into the minor groove of the formed DNA duplex and stabilizes the probe hybridization. Hence, MGB probes can be shorter (13–20 bases), and still possess similar melting temperatures as TaqMan probes [8]. In MGB probes the standard quencher TAMRA has been replaced by a non-fluorescent quencher (NFQ) acting as energy transfer acceptor from the reporter dye, but not emitting a fluorescent signal of its own. This results in a lower fluorescent background, improving the spectral discrimination and the ease of multiplexing [9].

Claimed advantages of these short probes are improved sensitivity towards single-base mismatches (specificity), ease of design and an improved signal-to-noise ratio, partly due to reduced fluorescence from spurious binding and partly due to the fact that the quencher is in closer proximity to the reporter dye [8].

The uni-molecular Scorpion has a Molecular Beacon tail linked to the 5′-end of the primer via a PCR-blocker. This probe anneals to the template DNA, and the DNA polymerase extends it, creating a complementary probe site. Following the next denaturation, the probe element will fold up and anneal to this site, rather than entering the hairpin loop conformation again, because it will be more kinetic favourable [10]. This event opens the hairpin loop, eliminating the quenching of the fluorophore and increasing the real-time PCR signal. The bi-molecular Scorpion consists of a fluorescently labeled probe coupled to a primer by a PCR-blocker. It is annealed to a complementary oligo bearing a quencher at the 3′-end. The mechanism of action is similar to that of the uni-molecular, the probe will anneal to the newly synthesised DNA

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Table 1
Primers and probes for detection of thermotolerant *Campylobacter* by real-time PCR.

Primer/Probe	Sequence	Bases	T_m [°C][e]	Manufacturer
OT-1559	CTGCTTAACACAAGTTGAGTAGG	23	55.7	DNA Tech.
18-1	TTCCTTAGGTACCGTCAGAA	20	54.6	DNA Tech.
TaqMan	FAM-TGTCATCCTCCACGCGGCGTTGCTGC-TAMRA	26	77.9	DNA Tech.
MGB	FAM-TGTCATCCTCCACGCGG-MGB-NFQ	17	69.0	ABI
LNA[a]	FAM-CA + TCC + TCCACGCGGCG + TTGC-BHQ1	20	76.0	Sigma–Proligo
Scorpion[b] R_Sc1	FAM-CCGCGTGGAGGATGAC-HEG-TTCCTTAGGTACCGTCAGAA	37	68.9	DxS
Scorpion[c] P1_Q	GTCATCCTCCACGCGG-Dabcyl	16	50.9	DxS
Scorpion[d] R_Sc2	FAM-CCCGCGTGGAGGATGACGCGGG-Dabcyl-HEG-TTCCTTAGGTACCGTCAGAA (Uni-molecular)	44	74.2	DxS

DNA Tech.: DNA Technology A/S, Århus, Denmark; ABI: Applied Biosystems, Cheshire, UK; Sigma–Proligo: Proligo France SAS, Paris, France; DxS: DxS Ltd., Manchester, UK.
[a] The + in front of the nucleotide indicates an LNA monomer substitution.
[b] Reverse primer linked together with the fluorescent probe (bi-molecular format).
[c] Separate oligo labeled with a dark quencher (bi-molecular format).
[d] Reverse primer linked together with the probe element (a Molecular Beacon tail labeled with a reporter and quencher dye at each end, uni-molecular format).
[e] The melting temperature of the probes and primers as stated by the manufacturers.

template, and emit fluorescence since it is separated from the quencher [3,10,11].

In this study, a statistically based comparison analysis was performed to identify the optimal probe chemistry for an already validated real-time PCR assay for detection of thermotolerant *Campylobacter*. The assay is intended for use in industrial routine testing of whole chicken carcass rinse samples, and therefore a high level of robustness and sensitivity is required.

A 5′-nuclease real-time PCR method for the specific detection of thermotolerant *Campylobacter* was performed essentially as described earlier [12] on an Mx3005P (Stratagene, La Jolla, CA). Fluorescence measurements were obtained online and analyzed with the MxPro-Mx3005P software (version 3.00). The threshold was assigned using the software option: background-based threshold, i.e. the standard deviation of all amplifications was determined from cycle 5 to 9, and this value was multiplied by a background sigma multiplier of 10.

(i) Each of the five probes (Table 1) was applied under standardized PCR conditions in the following concentrations: 50, 75, 100, 200, 250, 300, 400 and 500 nM in real-time PCR on triplicates of 3–3 × 10^5 copies/PCR of reference DNA (*C. jejuni* CCUG 11284). The parameters applied to evaluate the optimal probe concentration were cycle threshold (Ct)-values, background fluorescence, dR last (final fluorescence reading − initial fluorescence reading) and finally the variation between the triplicates.
(ii) The three probes (TaqMan, MGB and LNA) were run in the concentration determined as optimal in real-time PCR on triplicates of 3–3 × 10^5 copies/PCR reference DNA (*C. jejuni* CCUG 11284). The parameters applied to evaluate the performance of the probes were as mentioned above, including amplification efficiency (AE) calculated as: $AE = 10^{(-1/slope)} - 1$ [13].
(iii) The sensitivity of the TaqMan and the LNA probes was examined by performing replicate PCR on different levels of DNA copies. As shown in Table 2, multiple replicates of PCR were tested on 3, 10, 15, 30, 50, 100, 150, 300, 3000, 30,000, 300,000 and 3,000,000 copies/PCR of reference DNA (*C. jejuni* CCUG 11284). The relationship between the proportion positive from each replicate and the number of DNA copies of *C. jejuni* was estimated by analysis in a generalized linear model with a logit link function for the LNA and the TaqMan probes using the SAS software (SAS Institute, Cary, NC, USA). The dependency of the DNA level was described by a regressor.

The best performance of the TaqMan probe was obtained applying a 100-nM concentration. At this concentration, the lowest Ct-values and background fluorescence and the best splitting of the probe were observed. The MGB probe had an equally optimal performance at concentrations of 200 and 250 nM, where the Ct-values were the lowest and the background fluorescence was in an acceptable range. The best splitting of the probe was observed at 200 nM. Applying the LNA probe at a concentration of 75 nM gave the lowest Ct-values, acceptable background fluorescence and an optimal splitting of the probe. The Scorpion chemistry, in both the uni- and bi-molecular format, was not compatible with the real-time PCR assay. Non-amplification related degradation of the probe element was observed from the very first PCR cycle, until it reached plateau. Theoretically, the Scorpion chemistry should be more sensitive than the other probes tested, since the probing mechanism is intra-molecular, and the quenching mechanism predominantly collisional in contrast to TaqMan, LNA and MGB probes that rely on 'through-space' quenching [14]. The PCR products obtained when Scorpion probes were applied were run in gel electrophoresis (1.5% agarose gel, 100 V, 30 min), and the presence of correct-sized bands was observed. Several optimization steps were performed as recommended by the manufacturer (David Withcombe, DxS Ltd., personal communication), i.e. the amount of $MgCl_2$ was altered, the probe was run in a pre-fabricated master mix (DyNAmo qPCR Kit, Finnzymes, Espoo, Finland), with a different DNA polymerase (DyNAzyme, Finnzymes) and finally the thermal profile was optimized. However, none of the optimization steps rectified the problem of probe degradation.

As shown in Table 3, the comparison of the three probes at optimal concentrations revealed that the LNA probe produced slightly lower Ct-values than the TaqMan probe and especially the MGB probe. The Ct-values obtained by the LNA probe was significantly ($P < 0.05$, analysis of variance) lower than those obtained by

Table 2
Number of replicate PCR analysis performed and the number of positives with the LNA and the TaqMan probes at the different levels of DNA copies from *C. jejuni* CCUG 11284.

DNA copies/PCR	Total no. of replicates	No. of positives	
		LNA probe	TaqMan probe
3	30	0	0
10	20	1	0
15	20	6	1
30	46	43	23
50	10	9	10
100	30	30	28
150	30	30	30
300	46	46	46
3,000	36	36	36
30,000	6	6	6
300,000	6	6	6
3,000,000	6	6	6

Table 3
Cycle threshold (Ct)-values and amplification efficiencies obtained comparing the three probe types at optimal concentrations analyzing triplicate samples.

DNA [copies/PCR]	TaqMan [100 nM]	MGB [200 nM]	LNA [75 nM]
3×10^5	18.83	19.40	18.33
	18.50	19.34	18.52
	18.34	18.80	17.59
3×10^4	22.73	23.13	22.05*
	22.79	22.60	21.53*
	22.91	22.48	21.49*
3×10^3	26.86	27.21	26.72*
	26.84	26.85	25.60*
	26.80	27.51	26.08*
3×10^2	30.78	31.99	30.87**
	30.72	31.84	30.24**
	30.64	31.76	30.34**
3×10^1	34.37	35.44	33.47*
	34.27	35.24	33.11*
	34.89	35.68	33.15*
Amplification efficiency	0.786	0.740	0.804

*The Ct-values were significantly (analysis of variance, $P < 0.05$) lower using the LNA probe.
**The Ct-values were significantly (analysis of variance, $P < 0.05$) lower using the LNA and the TaqMan probe.
The Scorpion chemistry, in both the uni- and bi-molecular format, was not compatible with the real-time PCR assay.

the MGB probe for 4 out of 5 DNA levels. The background fluorescence of the LNA probe (16,000–34,000 fluorescence units) was higher than both that of the TaqMan (12,000–20,000) and MGB probe (14,000–21,000), and the splitting of the LNA probe was slightly inferior (approx. 3% less than the TaqMan and MGB probes). The variation between the triplicates was negligible for all three probes. The highest amplification efficiency was however achieved using the LNA probe.

The statistical model applied for the sensitivity analysis accounted for the nature of the data setup by adding a variable dividing the amount of DNA copies in to two levels, i.e. a low level up to 150 DNA copies, and a high level above 150 DNA copies. The results of the sensitivity analysis showed that the LNA probe was significantly ($P = 0.0001$) more sensitive than the TaqMan probe analyzing low levels of DNA copies, and that the proportion of positive responses depended on the level of DNA (Table 2). In the high level (>150 DNA copies) no difference in sensitivity was observed between the two probe types.

This is in accordance with the findings of Costa et al. (2004), who showed LNA probes to be more sensitive than conventional DNA probes testing fetal DNA in maternal serum samples. This was attributed to an improved PCR efficiency when using a shorter probe due to less amplification interference [15]. A comparative study on sensitivity formerly performed by the present authors (Reynisson et al. 2006), did likewise indicate an improved performance of an LNA probe compared with a TaqMan probe and a unimolecular Scorpion detecting *Salmonella* in a range of sample matrices. The LNA probe reached higher fluorescence plateaus, and produced lower Ct-values and standard deviations [16].

The advantages of the LNA probes are similar to MGB probes. They both belong to the short probe types with a high thermal duplex stability, and they are both reported to have an improved signal-to-noise ratio due to reduced signal from spurious binding and reduced distance between the fluorophore and quencher. However, the MGB probe was not found to be superior to the TaqMan probe in this study. Nevertheless, the sensitivity of an LNA probe was reported similar to that of an MGB probe by Letertre et al. (2003) amplifying enterotoxin genes from *Staphylococcus aureus* [17]. In PCR testing for hepatitis B by Zhao et al. (2005) also found an MGB probe to have a wider linear detection range and a lower limit of detection than a TaqMan probe [18]. As the data and discussion presented above emphasizes, it is necessary to evaluate the optimal probe type for each PCR assay separately, as conclusions drawn from one comparison study are not necessarily generally applicable.

In conclusion, optimizing the probe chemistry is a convenient way to improve sensitivity of a PCR assay. In the assay evaluated in this study, the Ct-values were improved when using an LNA probe and higher amplification efficiency was observed. The LNA probe was likewise proven to be the most sensitive of the chemistries. This probe chemistry presents an interesting alternative to TaqMan probe-based real-time PCR assays, not least due to its sensitivity towards single-base mismatches, ease of design and improved signal-to-noise ratio.

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Quantification of viable *Campylobacter* in fresh chicken carcass rinse by real-time PCR and propidium monoazide as a novel strategy for risk assessment. Josefsen, M.H., C. Löfström, T.B. Hansen, L.S. Christensen, J.E. Olsen and J. Hoorfar.

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Quantification of viable *Campylobacter* in fresh chicken carcass rinse by real-time PCR and propidium monoazide as a novel strategy for risk assessment

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Abstract

Mitigation strategies for *Campylobacter* are focusing increasingly on post-slaughter reduction of the number of cells, emphasizing the need for rapid and reliable quantitative detection of *only* viable *Campylobacter*. We present a quantitative real-time PCR (Q-PCR) method for detection and enumeration of foodborne thermotolerant *Campylobacter* (*C. jejuni*, *C. coli* and *C. lari*), combined with a simple propidium monoazide (PMA) sample treatment, which generates signal from only viable and viable but non-culturable (VBNC) *Campylobacter* with an intact membrane in chicken carcass rinse. The performance of the method was evaluated by assessing the contribution to variability from individual chicken carcass rinse matrices, different species of *Campylobacter*, and the efficiency of DNA extraction with differing cell inputs. Finally, the method was compared to culture-based enumeration on 50 naturally infected Danish chickens from a *Campylobacter*-positive flock. The amplification efficiency of the Q-PCR method was found to be unaffected by chicken rinse matrix and species of *Campylobacter*. The relationship between cell input and Ct-value was linear (R^2 = 0.993), with a quantification range from 1×10^2 - 1×10^7 CFU/ml. The correlation between the *Campylobacter* counts obtained by PMA-PCR and culture on naturally contaminated chicken carcass rinse samples was good (R^2 = 0.8436). In conclusion, this study presents a reliable diagnostic tool for producing accurate quantitative data on viable *Campylobacter* in chicken carcass rinse for risk assessment. The proposed method does not detect DNA from dead Campylobacter, but recognises the infectious potential of the VBNC state, and is thereby able to assess the outcome and impact of new mitigation strategies.

Introduction

As *Campylobacter* remains the leading cause of food borne bacterial gastrointestinal disease in large parts of the developed world (30), affecting general health and having wide socioeconomic impact, much effort is devoted to develop and improve detection methods. The objective of this is partly to be able to supply consumers with fresh *Campylobacter*-free products, and perhaps more importantly, to gain more insight in the epidemiology of *Campylobacter* in order to improve mitigation strategies and make them more efficient.

Traditional culture-based detection of *Campylobacter* including enrichment, isolation and confirmation is a time-consuming procedure requiring 5 to 6 work days (3, 12). Another drawback of culture-based detection of *Campylobacter* is that these bacterial cells may enter a viable but non-culturable (VBNC) state, in which their potential to cause human infection is controversial (32). The introduction of the quantitative real-time PCR (Q-PCR) technology has enabled a more rapid, sensitive and less work demanding quantitative detection. Q-PCR methods for food borne *Campylobacter jejuni* and *C. coli* in poultry, which is recognized as an important source of human *Campylobacter* infections, have been published (9, 10, 13, 33, 39). However, bearing in mind that the majority of current mitigation strategies regarding *Campylobacter* are focused on post-slaughter reduction of the number of bacterial cells on the chicken carcass, the usefulness of these Q-PCR methods for risk assessment could be limited, since they detect all *Campylobacter* present in a sample, including the dead cells.

The Q-PCR method described in the present study quantifies the three major foodborne *Campylobacter* species (*C. jejuni*, *C. coli* and *C. lari*), thereby covering all possible prevalence shifts and co-infections. The method was previously validated according to the Nordic organization for validation of alternative microbiological methods (NordVal), and is certified for detection of *Campylobacter* in chicken, cloacae swabs and boot swabs. The current study confirms its suitability for quantification of *Campylobacter* in chicken carcass rinse. Furthermore, a propidium monoazide (PMA) sample treatment step has been incorporated into the method (PMA-PCR), assuring quantification of only viable *Campylobacter* cells with intact membrane. Propidium monoazide is a chemical alteration (additional azide group) of propidium iodide (PI), one of the most frequently applied membrane-impermeable dyes in flow cytometry, and it can be expected to have the same permeability potential as PI. This is of great value in a food safety perspective, since PI only penetrates permeabilized cells, and not the cells with intact membranes (including the *Campylobacter* VBNC state), which can be resuscitated and therefore hold the potential of

infection (26). This study, for the first time, establishes the correlation between PMA-PCR and culture for a large number of naturally infected Danish broilers.

Materials and Methods

Experimental design

Preliminary optimization and investigation of the quantitative aspect: Preliminary experiments were performed to determine the (i) optimal volume (50, 100 or 500 ml) applied for rinsing of chicken carcasses, (ii) optimal volume to draw from the chicken carcass rinse (1, 5 or 10 ml) for DNA extraction (iii) if DNA extraction was needed or if Q-PCR could be performed directly on chicken carcass rinse and finally (iiii) optimal DNA template volume (5 or 10 μl) for Q-PCR.

Furthermore, the recovery of *Campylobacter* with the applied rinsing procedure was examined. Firstly, two *Campylobacter*-positive chickens were purchased at retail level and rinsed as described, four times in succession. From each chicken carcass rinse, ten-fold serial dilutions ranging from 10^0 to 10^{-2} were prepared, and the number of *Campylobacter* was determined in duplicate by culture as described. Secondly, eight *Campylobacter*-free chickens were purchased at retail level and split in half. From one half, the *Campylobacter*-negative status of the chicken was confirmed by culture of the chicken carcass rinse as described. The second halves were inoculated with 5×10^2, 5×10^3, 5×10^4 and 5×10^5 CFU *C. jejuni* CCUG 11284. The inoculation was performed by placing 10 μl droplets of bacterial suspension at 25 equally distributed positions on the chicken skin surface. To ensure adherence and simulate natural contamination as far as possible, the chicken was placed unwrapped for 1 hour at room temperature followed by 24 hours at 5°C wrapped in a sterile plastic bag. Subsequently, the number of *Campylobacter* on the inoculated chickens was determined by culture of the chicken carcass rinse as described.

Variation attributable to the matrix: A prerequisite for successful quantification of *Campylobacter* is to describe the natural variation within the matrix in question. Quantification would be flawed and encumbered with uncertainties if large natural variation was observed in the chicken carcass rinse matrix. In order to evaluate this variation, twelve *Campylobacter*-free chickens of mixed origin were purchased from local retailers. They were rinsed as described, and the rinse was inoculated with ten-fold dilutions ranging from 1×10^2 to 1×10^6 CFU/ml *C. jejuni* and *C. coli*, respectively, and subsequent DNA extraction and Q-PCR was performed.

Variation attributable to the individual species: The Q-PCR assay described in this study amplifies the three foodborne species of *Campylobacter*: *C. jejuni*, *C. coli* and *C. lari*. In order to quantify all species simultaneously, without further differentiation, equal sensitivity and amplification efficiency (AE) for all species had to be assumed. To test this hypothesis, standard dilution series

made from both pure DNA and bacterial cells of the three species were investigated by Q-PCR analysis.

The DNA concentration of the following strains: *C. jejuni* CCUG 12795 and 14539, *C. coli* CCUG 10951 and 12791 and *C. lari* CCUG 20707 and 23947 was adjusted to 1 ng/µl based on measurements performed on a NanoDrop (Thermo Scientific, Waltham, MA), and ten-fold serial dilutions were prepared and analysed in duplicate by Q-PCR (100 fg-10 ng/PCR).

Campylobacter jejuni CCUG 11284, *C. coli* CCUG 11283 and *C. lari* CCUG 23947 were recovered on five blood agar plates each. For each species, the growth was transferred to approx. 10 ml of physiological saline, and the suspension was mixed thoroughly to ensure a homogenous suspension of bacterial cells. A ten-fold serial dilution was prepared for each species ranging from 10^{-1} to 10^{-9}. The numbers of bacterial cells in the suspensions were determined by duplicate plate spreading on blood agar. Standard rows ranging from 1 to 1×10^{6} CFU/ml were produced from the three species. To improve the linearity of the standard curves a background of 10^{8} to 10^{9} CFU/ml *Escherichia coli* ATTC 25922 was added to the standards. One ml volumes were drawn from the standards, DNA was extracted as described and they were analysed in duplicate by Q-PCR. This procedure was repeated with two biological replicates, four times for *C. jejuni*, and two times for *C. coli* and *C. lari*, to observe the variation between different bacterial suspensions and dilution series.

Quantification of live *Campylobacter* in naturally infected Danish chickens post slaughter: The number of *Campylobacter* on fifty chickens from a confirmed *Campylobacter*-positive flock was quantified in parallel by Q-PCR (with and without PMA sample treatment) and conventional culture. The chickens were collected immediately after cooling at the abattoir, packed in sterile plastic bags, and kept at 5°C until analysed (< 18 h). The chickens were rinsed as described. Duplicate chicken carcass rinse volumes, with and without PMA treatment prior to DNA extraction, were analysed by Q-PCR in duplicate as described. From parallel duplicate chicken carcass rinse volumes, ten-fold serial dilutions ranging from 10^{0} to 10^{-2} were prepared and the number of *Campylobacter* was determined in duplicate by culture as described.

Bacterial strains and culture conditions

Campylobacter strains were stored at -80°C in LB medium containing 15% glycerol as cryoprotectant (Statens Serum Institute, SSI, Copenhagen, Denmark). They were recovered on blood agar (SSI) and isolated on selective solid medium; modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid, Greve, Denmark) and Abeyta-Hunt-Bark agar (AHB, Technical

University of Denmark, DTU, Copenhagen, Denmark). Mueller-Hinton broth (SSI) was used to produce overnight cultures for preparing the quantification standards, and for artificial inoculation of chicken carcass rinse. Incubation was at all times performed at 41.5±1°C under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2 and 80% N_2).

Chicken carcass rinse

Chicken carcass rinse was prepared according to the recommendations in ISO 6887-2 (6). A whole fresh chicken was placed in a sterile plastic bag and rinsed manually in 50 ml of physiological saline (0.9% NaCl) for 1 min. One ml aliquots were drawn for DNA extraction and subsequent Q-PCR analysis, and for culture.

Culture-based enumeration

From the chicken carcass rinse duplicate ten-fold serial dilutions ranging from 10^0 to 10^{-2} were prepared and 100 µl of each dilution was spread onto mCCDA and AHB and incubated for 48 h. The agar plates were dried for 30 min prior to use, to avoid swarming of the colonies. Two selective agar plates were applied according to the recommendations of ISO 10272-1. Five presumptive *Campylobacter* colonies pr. chicken carcass rinse were verified by colony-PCR (20), by dissolving a minimal amount of colony material in 100 µl of physiological saline and analyzing 10 µl of this suspension in Q-PCR, with 10 min of primary denaturation to ensure cell wall disruption and DNA accessibility.

Propidium monoazide treatment of samples

Preliminary experiments and literature reviews were performed to determine the concentration of PMA, time of incubation and light exposure, and distance from light source. Propidium monoazide (PMA, Biotium Inc., Hayward, CA) dissolved in 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Brøndby, Denmark) was added to 1 ml volumes of chicken carcass rinse to a final concentration of 10 µg/ml. The chicken carcass rinse added PMA was incubated in clear Eppendorf tubes in the dark for 5 min, and inverted repeatedly twice during this period. Following incubation the Eppendorf tubes were placed on ice and exposed to a 650 W halogen light source (Kaiser Videolight 6, Kaiser Fototechnik, Buchen, Germany), with a distance of 20 cm, for 1 min. The Eppendorf tubes were swirled shortly by hand every 15 sec, and turned over after 30 sec of illumination, to ensure complete cross linking of available DNA, and conversion of free PMA to hydroxylamino propidium.

DNA extraction

One ml volumes of chicken carcass rinse/standard were centrifuged at 3000 x *g* for 5 min at 4°C, and DNA-extraction performed on a KingFisher (Thermo Labsystems, Helsinki, Finland) using a DNA isolation kit for blood, stool, cells and tissue (Magnesil KF, Genomic system, Promega) as specified by the manufacturer. Briefly, the sample pellet was re-suspended in 200 μl lysis buffer and transferred to wells A, B and C of a 96-well plate (Thermo Labsystems) containing paramagnetic particles (90 μl divided into wells A, B and C), salt washing buffer (200 μl divided into wells D and E), alcohol wash (200 μl divided into wells F and G) and 100 μl of elution buffer (well H), and the DNA extraction program (Genomic_DNA_1) was performed. Ten μl of the extracted DNA was used as template in the Q-PCR. In every 96-well plate (corresponding to 12 DNA extractions) a process control consisting of only DNA extraction reagents was included.

Q-PCR analysis

A 5' nuclease Q-PCR method for the specific detection of foodborne thermotolerant *Campylobacter* was performed on an Mx3005P (Stratagene, La Jolla, CA), as described previously (16) with the following modifications: 0.6 mM deoxynucleoside triphosphate mixture (Applied Biosystems, Naerum, Denmark), 0.5 μM forward and reverse primer, 0.2 g/L bovine serum albumin (BSA, Roche A/S), 0.8 ml/L glycerol (87%, Merck, Darmstadt, Germany), 75 nM target locked nucleic acid (LNA) *Campylobacter* probe 5' [6FAM] CA[+T] CC[+T] CCA CGC GGC G[+T]T GC [BHQ1] 3' (Sigma-Aldrich), 60 nM internal amplification control (IAC) probe, and 10 μl of extracted template DNA. The cycle profile was as follows: initial denaturation at 95°C for 3 min (10 min for colony-PCR), followed by 40 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 30 s. Fluorescence measurements were obtained online and analyzed with the MxPro-Mx3005P software (version 3.00). The threshold was assigned using the software option: background-based threshold, i.e. the standard deviation of all amplifications was determined from cycle 5 to 9 (or a similar appropriate interval), and this value was multiplied by a background sigma multiplier of 10.

In every Q-PCR analysis the *C. jejuni* standard for absolute quantification was included in duplicate. Furthermore, a non-template control (NTC) containing PCR grade water, a negative DNA control (5 ng of *Escherichia coli* DNA) and a positive DNA control (5 ng of *Campylobacter jejuni* DNA) were included.

Standard curve for absolute quantification

Campylobacter jejuni CCUG 11284 was recovered on blood agar (SSI). A loop full of colony material was transferred to 10 ml of Mueller-Hinton broth and incubated for approx. 16 hours. From this suspension a ten-fold serial dilution was prepared and enumerated by plate spreading onto blood agar in triplicate. One ml volumes of *Campylobacter*-free chicken carcass rinse were inoculated with 1×10^2 to 1×10^7 CFU *C. jejuni* CCUG 11284 from the appropriate dilution, and the DNA extracted from these as described above. Three biological and six Q-PCR replicates was used to produce the standard curve.

Data analysis

Variation attributable to matrix and species: The variation between the standard curves produced in these experiments was evaluated by linear regression (Microsoft® Excel 2000). For each standard curve, CFU/ml or DNA concentration was $\log_{10}$-transformed and defined as the independent variable. The Ct- or ΔCt-value was defined as the dependent variable. The 95% confidence intervals of estimates of slope and intercept, respectively, were used for the comparison of standard curves. Standard curves with overlapping 95% confidence intervals were not regarded as statistically significantly different.

Standard curve: The standard curve was produced by plotting the Ct-value obtained in Q-PCR against the number of CFU in the standards. From this linear relationship the AE was calculated from: $AE = 10^{-1/slope}-1$ (19).

Quantification of live *Campylobacter* in Danish chickens post slaughter: The correlation between the results obtained by Q-PCR (with and without PMA-treatment) and the culture-based enumeration was evaluated by plotting the $\log_{10}$-transformed *Campylobacter* cell equivalents (CCE)/ml obtained from the Q-PCR against the $\log_{10}$-transformed CFU/ml obtained by duplicate plate spreading on mCCDA. For comparison of the ability of Q-PCR (with and without PMA) to predict CFU counted on mCCDA, the difference between the $\log_{10}$-transformed bacterial numbers was used as response variable in a single factor analysis of variance (Microsoft® Excel 2000), comparing Q-PCR with PMA to Q-PCR without PMA. A *P*-value below 0.05 was considered statistically significant.

Results

Preliminary optimization and investigation of the quantitative aspect: Preliminary experiments were performed to optimize the volume applied for rinsing of chicken carcasses, the volume to draw from the chicken carcass rinse for DNA extraction and the DNA template volume for Q-PCR. Optimal method performance was obtained when a rinse volume of 50 ml, a sampling volume of 1 ml, and a DNA template volume of 10 µl was applied (data not shown). Furthermore, it was not possible to obtain good detection without the DNA extraction step.

Experiments to evaluate the recovery of Campylobacter in the 50 ml rinsing procedure were performed. On naturally infected chicken carcasses, the second rinsing procedure yielded approx. 60% of the initial amount of *Campylobacter* than the initial rinsing. Performing two subsequent rinsing procedures 45%, and 27% of the initial amount of *Campylobacter* were recovered. The recovery of *Campylobacter* from artificially inoculated chicken carcasses ranged from 55 to 94% and was determined to be 77% in average.

Variation attributable to the matrix and individual species: The variation attributable to the chicken carcass rinse matrix was evaluated from twelve standard curves of *C. jejuni* and *C. coli* in different chicken carcass rinse matrices. As shown in Figure 1, the contribution to variation from the chicken carcass rinse matrix was negligible, as no statistical significant difference ($P > 0.05$) was found.

The variation attributable to the individual *Campylobacter* species was evaluated from standard curves of both pure DNA and bacterial cells. Standard curves produced by dilution of DNA from six individual strains were constructed (Figure 2), and it was found that the variation between species was statistically insignificant ($P > 0.05$). Standard curves for cells were obtained by subtracting the Ct-value for each dilution from the Ct-value obtained for the highest number of cell input (Figure 3). Delta Ct-values were applied to describe the variation between the three species on cell level since the input cell number was difficult to accurately standardize, and this could obscure the true variation, Similarly to the DNA, the variance between the three species was not significant ($P > 0.05$) on the cell level, proving equal sensitivity and amplification efficiency of the method independent of species.

Standard curve for absolute quantification using Q-PCR: The standard curve, produced from DNA extracted from 10-fold cell dilutions in chicken carcass rinse, applied for absolute quantification of *Campylobacter* in the naturally infected chicken carcass rinse showed an AE of 91%, computed from the slope of the linear relationship between the $\log_{10}$-transformed CFU/ml and the Ct-value

(R^2 = 0.993). The method was shown to be linear in the range of 1×10^2 to 1×10^7 CFU/ml chicken carcass rinse, and the limit of quantification thereby 1×10^2 CFU/ml. Taking into consideration the volume reduction during DNA extraction, this corresponds to 10 CFU/PCR. From the standard curve, the level of *Campylobacter* was expressed by the equation: Ct = -3.571×Log (CFU) + 40.8.

Evaluation of variation in culture based enumeration: Two parallel dilutions of chicken carcass rinse was plate spread onto selective agar (mCCDA and AHB), to evaluate the contribution of the culture enumeration to the overall variation of the method. The linear relationship between the replicate culture series was expressed by the equations: y = 1.0004x for mCCDA (R^2 = 0.9693) and y = 1.0154x for AHB (R^2 = 0.9351), indicating an extremely low variation in the quantitative results obtained by culture.

Quantification of live *Campylobacter* in naturally infected Danish chickens post slaughter: The number of *Campylobacter* on fifty chickens post slaughter from a confirmed *Campylobacter*-positive flock was quantified in parallel by Q-PCR (with and without PMA sample treatment) and conventional culture (Table 1). By culture, 42 chickens were found *Campylobacter*-positive, while this number was 45 for Q-PCR with PMA-treatment, and 48 without PMA-treatment. The culture-negative/Q-PCR-positive chickens had low levels of *Campylobacter*, below the quantification limit of the Q-PCR. The same applied for chickens found *Campylobacter*-positive by Q-PCR without PMA-treatment, but *Campylobacter*-negative with PMA-treatment. The infection level of the *Campylobacter*-positive chicken carcass rinses ranged from 25 to 1.5×10^6 CFU/ml, reflecting the difference in contamination in a flock.

The correlation between the results obtained by Q-PCR (with and without PMA-treatment) and the culture-based enumeration was evaluated. Except for chicken no. 2 and 7, where the CCE/ml were approximately the same with and without PMA-treatment, the PMA-treated samples gave a reduced signal in Q-PCR. A reduction-range of 1×10^2 to 2.4×10^6 CCE/ml was observed in PMA-treated samples, and the reduction was positively correlated to the amount of *Campylobacter* in the samples. This is also reflected in the obvious linear correlation between the Q-PCR results obtained from both PMA-treated and -untreated samples and the enumeration by culture (Figure 4). The data analysis of the culture-based enumeration was based on the average obtained from the duplicate mCCDA plates, since a better correlation was obtained by these, than the AHB plates. Analysis of variance comparing Q-PCR (with and without PMA) to plate counts on mCCDA, showed that Q-PCR *with* PMA-treatment produced results that were statistically significantly closer to counts on mCCDA ($P < 0.001$).

Discussion

As many advantages as the Q-PCR technology offers regarding pathogen detection in mixed populations, a major drawback has been that PCR detection, operating on DNA level, cannot distinguish between DNA arising from viable and dead bacterial cells. This has been one of the main causes hindering the implementation of PCR in routine diagnostics for food, where quantification of viable bacteria is essential in relation to risk assessment. It is a serious limitation of the PCR technology which is of particular relevance for *Campylobacter*, since mitigation strategies are focusing increasingly on post-slaughter decontamination, leaving the PCR technology unable to assess the potential for foodborne infections. In 2006, EC Regulation (No. 853/2004) permitted the use of other substances than water for decontamination of meat surfaces (5). Both physical and chemical reduction strategies, including treatment with steam, ultraviolet light, high hydrostatic pressure, essential oil fractions, acid and sodium hypochlorite, have been investigated for their *Campylobacter* reduction potential, with varying outcome (1, 7, 8, 11, 14, 21, 25, 29, 31). DNA from dead bacterial cells has been shown to persist for up to three weeks following cell death (18), and it has also been demonstrated that the presence of DNA from dead *Campylobacter* cells can lead to an overestimation of the number of viable cells, and in some instances, even to false positive responses (37).

In the present study a novel technique to distinguish between viable and dead cells by PMA-PCR (27, 28, 35), was applied successfully to diagnostic quantification of viable *Campylobacter* in naturally contaminated chicken carcass rinse. PMA-PCR is claimed to detect “viable” cells. However, “viable” can be defined in several ways: as having an intact membrane, the ability to metabolise compounds and/or the ability to withhold a proton gradient between the inner and outer part of the cell. To further distinguish between these states the PMA-PCR method can be supplemented with other compounds, e.g. to show the presence of an intact metabolism (27). Despite this uncertainty, the PMA-PCR quantification compared favourably to direct culture-based detection of *Campylobacter* in this study. The relative specificity of the PMA-PCR method was 100%, and it was shown to be more sensitive than the culture-based method. Three chicken carcass rinse samples were found positive by PMA-PCR but not by culture. According to ISO 20838:2006 these can be regarded as true positives due to the target specific DNA-probe based PCR-response (4). Besides being labour-intensive and time-consuming, culture-based quantification will not detect the VBNC fraction of a given *Campylobacter* population. The results obtained in the present study, suggest that VBNC *Campylobacter* were not present in great numbers on the chicken carcasses sampled immediately post slaughter. However, the ratio between viable and VBNC cells can only be expected to decrease as a consequence of food processing and storage, thus presenting a possible diagnostic uncertainty. Furthermore, post

slaughter mitigation strategies to reduce viable *Campylobacter* on chicken carcasses, will most certainly contribute to the number of VBNC cells. As long as the infectious potential of the *Campylobacter* VBNC state is not clarified, quantitative methods for risk assessment should detect these as well.

The good correlation demonstrated in this study between the counts obtained by Q-PCR with PMA-treatment and culture, enables calculation of the amount of *Campylobacter* in naturally infected chicken carcass rinse from a Ct-value obtained by Q-PCR. It has been demonstrated in several studies, comparing Q-PCR to culture-based enumeration, that higher counts are produced by Q-PCR, which have been explained by the detection of DNA from dead and VBNC cells (9, 13, 39). In the present study this was also true for the PMA-untreated samples, however when the PMA-treatment was applied a lower Q-PCR count than culture-based count was often observed. The reason for this could partly be due to underestimation of the cell input in the standard applied for quantification, but also due to overestimation of the number of *Campylobacter* colonies on the mCCDA plates. Five presumptive *Campylobacter* colonies from each chicken carcass rinse were sub-cultured to non-selective medium and subsequently verified by colony-PCR. The results indicated that not all *Campylobacter*-like colonies on the mCCDA plates could in fact be confirmed as being *C. jejuni*, *C. coli* or *C. lari*, substantiating this theory (data not shown). Another issue that has to be considered in this regard is that the cell state, and permeability of the cell wall, is not a clear cut reflection of a viable or dead cell, and PMA could have entered a minor fraction of culturable cells.

The quantification limit of the present PMA-PCR method was 100 CFU/ml chicken carcass rinse, not meeting the legislative demands of detection of 1 CFU/25 g (3). Available technologies do, however, not enable separation and concentration of the target organism from the food matrix, resulting in the necessity of a certain level of *Campylobacter* before direct detection by Q-PCR (and culture) is possible. The results from the preliminary rinsing experiments also showed that the low rinsing volume of 50 ml, did far from recover all the *Campylobacter* cells present on the chicken carcasses. The rinsing of artificially infected chickens resulted in an average recovery of 77%, but despite droplet inoculation and time for attachment to simulate natural infection, it is likely that recovery would be lower from natural infected chickens. A rinsing volume of 310 ml was applied in a study by Jørgensen et al. (2002), yet similar recovery rates to the present study were observed; 49 and 25% of the yield from the primary rinse was found in the second and third rinsing procedure (15). This has to be considered in estimating the whole carcass contamination level when employing the PMA-PCR method for enumeration of naturally contaminated chicken carcasses. Immunocapture of *Campylobacter* prior to PCR, reducing sample volumes from 250 ml to only 200 μl, have been conducted, but showed poor recovery rates (24). Wolffs et al. (2005)

described the use of flotation to single out viable and VBNC *Campylobacter* prior to Q-PCR. The limit of detection of this flotation-based method was determined to be 8.6×10^2 CFU/ml and quantification was possible in the range of 2.6×10^3 to 2.6×10^7 CFU/ml in chicken carcass rinse (38).

Despite not meeting the legislative detection demands, direct Q-PCR methods for *Campylobacter* are applicable as tools for risk assessment and assurance of food safety, since it has been shown that a strong positive correlation exists between the number of *Campylobacter* on chickens, and the risk of human infections (23, 34, 36). It would likewise be feasible that the risk of cross contamination is proportional to the number of *Campylobacter* cells present on the chicken carcass.

In contrast to other Q-PCR methods for *Campylobacter*, the assay applied in this study has been validated in comparative and collaborative trials according to the recommendations of the Nordic Organisation for Validation of Alternative Microbiological Methods (NordVal) (2) and approved for detection of *Campylobacter* in chicken, cloacae swabs and boot swabs (20). The inclusivity and exclusivity of the primers has been determined on 115 target and 87 non-target strains to 100 and 97%, respectively (22). The amplification efficiency, linear range, detection probability and detection precision of the assay has been evaluated and its suitability for quantitative PCR analysis confirmed (16). It includes in an internal amplification control to avoid false negative responses and reveal PCR inhibition. Finally, the standard dilution series for calculation of the level of *Campylobacter* was produced from ten-fold cell dilutions (not DNA) in the relevant matrix, from which DNA was subsequently extracted and analysed in Q-PCR, taking into account that efficiency of DNA extraction can vary substantially with initial amount of cells (17).

In conclusion, this study presents a diagnostic tool for quantitative detection of foodborne ***viable*** *Campylobacter*, that can be applied to produce accurate and reliable data for risk assessments in chicken carcass rinse. The Q-PCR-method is ready to be applied in industry and control laboratories, not detecting DNA from dead *Campylobacter*, but recognising the infectious potential of the VBNC state, and thereby able to assess the outcome and impact of new mitigation strategies.

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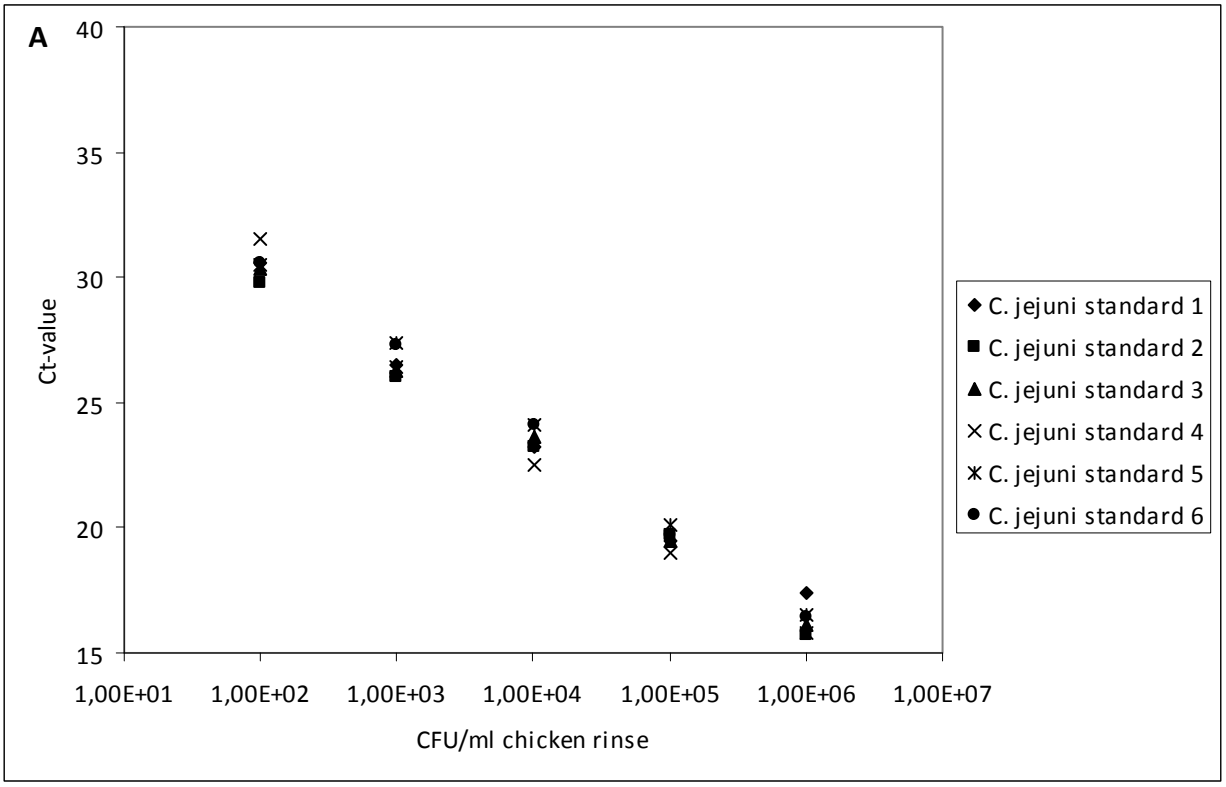
Table 1. Results obtained from Q-PCR (with and without PMA-treatment) and culture-based quantification of *Campylobacter* in naturally infected chicken carcass rinse.

	Q-PCR					Culture
	Ct-value *Campylobacter*		CCE *Campylobacter*/ml			
Chicken no.	w/ PMA	wo/ PMA	w/ PMA	wo/ PMA	Wo/ PMA-w/ PMA	CFU/ml
1	35.1	34.8	+	+	+	4.00×10^{1}
2	28.8	31.0	8.44×10^{2}	4.08×10^{2}	-4.36×10^{2}	2.55×10^{2}
3	30.5	30.1	2.70×10^{2}	3.75×10^{2}	1.05×10^{2}	3.90×10^{2}
4	21.6	22.1	1.20×10^{5}	1.94×10^{5}	7.42×10^{4}	1.17×10^{5}
5	30.7	31.9	2.26×10^{2}	+	+	3.85×10^{2}
6	28.1	28.4	1.39×10^{3}	2.67×10^{3}	1.28×10^{3}	1.90×10^{3}
7	25.8	27.1	6.68×10^{3}	6.52×10^{3}	-1.57×10^{2}	3.20×10^{3}
8	30.7	29.3	2.48×10^{2}	1.33×10^{3}	1.08×10^{3}	1.13×10^{3}
9	28.6	28.2	1.01×10^{3}	2.56×10^{3}	1.55×10^{3}	1.72×10^{3}
10	28.1	27.4	1.48×10^{3}	4.68×10^{3}	3.21×10^{3}	2.10×10^{3}
11	29.7	28.7	4.87×10^{2}	1.88×10^{3}	1.39×10^{3}	Swarming
12	21.1	20.2	1.74×10^{5}	6.56×10^{5}	4.81×10^{5}	2.07×10^{5}
13	28.5	28.1	1.11×10^{3}	2.95×10^{3}	1.84×10^{3}	7.30×10^{2}
14	30.0	28.2	3.96×10^{2}	2.59×10^{3}	2.19×10^{3}	1.00×10^{3}
15	27.3	25.5	3.33×10^{3}	1.67×10^{4}	1.33×10^{4}	2.90×10^{3}
16	29.3	27.8	6.29×10^{2}	4.20×10^{3}	3.57×10^{3}	8.20×10^{2}
17	31.0	28.8	2.02×10^{2}	1.81×10^{3}	1.61×10^{3}	3.00×10^{2}
18	28.0	26.0	1.48×10^{3}	1.14×10^{4}	9.90×10^{3}	1.41×10^{3}
19	17.9	17.6	1.53×10^{6}	3.97×10^{6}	2.44×10^{6}	$>2.00\times10^{4}$
20	29.5	28.2	5.62×10^{2}	2.98×10^{3}	2.42×10^{3}	7.95×10^{2}
21	23.6	23.4	3.07×10^{4}	7.34×10^{4}	4.27×10^{4}	1.37×10^{4}
22	38.2	+	+	+	+	Negative
23	Negative	Negative	Negative	Negative	Negative	Negative
24	28.1	27.9	1.39×10^{3}	3.98×10^{3}	2.58×10^{3}	6.35×10^{2}
25	31.3	30.2	1.58×10^{2}	7.19×10^{2}	5.61×10^{2}	7.45×10^{2}
26	25.0	24.7	1.15×10^{4}	3.07×10^{4}	1.92×10^{4}	8.95×10^{2}
27	35.8	39.0	+	+	+	Negative
28	Negative	+	Negative	+	Only + wo/ PMA[a]	Negative
29	Negative	+	Negative	+	Only + wo/ PMA[a]	Negative
30	28.5	27.9	1.07×10^{3}	3.37×10^{3}	2.29×10^{3}	1.19×10^{3}
31	26.0	24.9	5.85×10^{3}	2.42×10^{4}	1.83×10^{4}	4.70×10^{3}
32	29.3	28.0	6.21×10^{2}	3.03×10^{3}	2.41×10^{3}	9.85×10^{2}
33	25.3	24.0	9.48×10^{3}	4.61×10^{4}	3.66×10^{4}	1.70×10^{4}
34	31.3	29.4	1.61×10^{2}	1.14×10^{3}	9.83×10^{2}	7.15×10^{2}
35	28.2	26.9	1.42×10^{3}	6.28×10^{3}	4.87×10^{3}	4.40×10^{3}
36	26.4	25.1	4.35×10^{3}	2.39×10^{4}	1.95×10^{4}	1.09×10^{4}
37	28.1	27.5	1.42×10^{3}	4.40×10^{3}	2.99×10^{3}	3.30×10^{3}
38	28.3	27.0	1.32×10^{3}	5.93×10^{3}	4.61×10^{3}	2.00×10^{3}
39	29.6	34.9	6.13×10^{2}	+	+	Negative
40	32.5	31.7	+	2.40×10^{2}	+	2.50×10^{1}
41	Negative	Negative	Negative	Negative	Negative	Negative
42	Negative	+	Negative	+	Only + wo/ PMA[a]	Negative
43	29.0	28.4	7.67×10^{2}	2.29×10^{3}	1.52×10^{3}	1.75×10^{3}
44	33.8	30.5	+	5.34×10^{2}	+	1.95×10^{2}
45	28.2	28.4	1.36×10^{3}	2.30×10^{3}	9.42×10^{2}	9.20×10^{2}
46	23.2	23.0	3.91×10^{4}	9.57×10^{4}	5.65×10^{4}	2.70×10^{4}
47	27.5	25.9	2.08×10^{3}	1.47×10^{4}	1.26×10^{4}	3.15×10^{3}
48	27.1	26.3	2.82×10^{3}	1.16×10^{4}	8.75×10^{3}	1.90×10^{3}
49	27.7	26.2	1.85×10^{3}	1.28×10^{4}	1.10×10^{4}	4.70×10^{3}
50	27.4	27.4	2.38×10^{3}	4.52×10^{3}	2.15×10^{3}	2.05×10^{3}

w/ PMA: PMA-treatment prior to DNA extraction and Q-PCR
wo/ PMA: No PMA-treatment
CCE: *Campylobacter* cell equivalents
+: *Campylobacter*-positive below the quantification limit

Figure 1. Standard curves produced from ten-fold serial dilutions ranging from 1×10^2 to 1×10^6 CFU/ml chicken carcass rinse of **(A)**: *C. jejuni* CCUG 11284 and **(B)**: *C. coli* CCUG 11283 cells in six different chicken carcass rinses, showing the variation that can be ascribed to the matrix. Each data point represents three biological and six Q-PCR replicates.



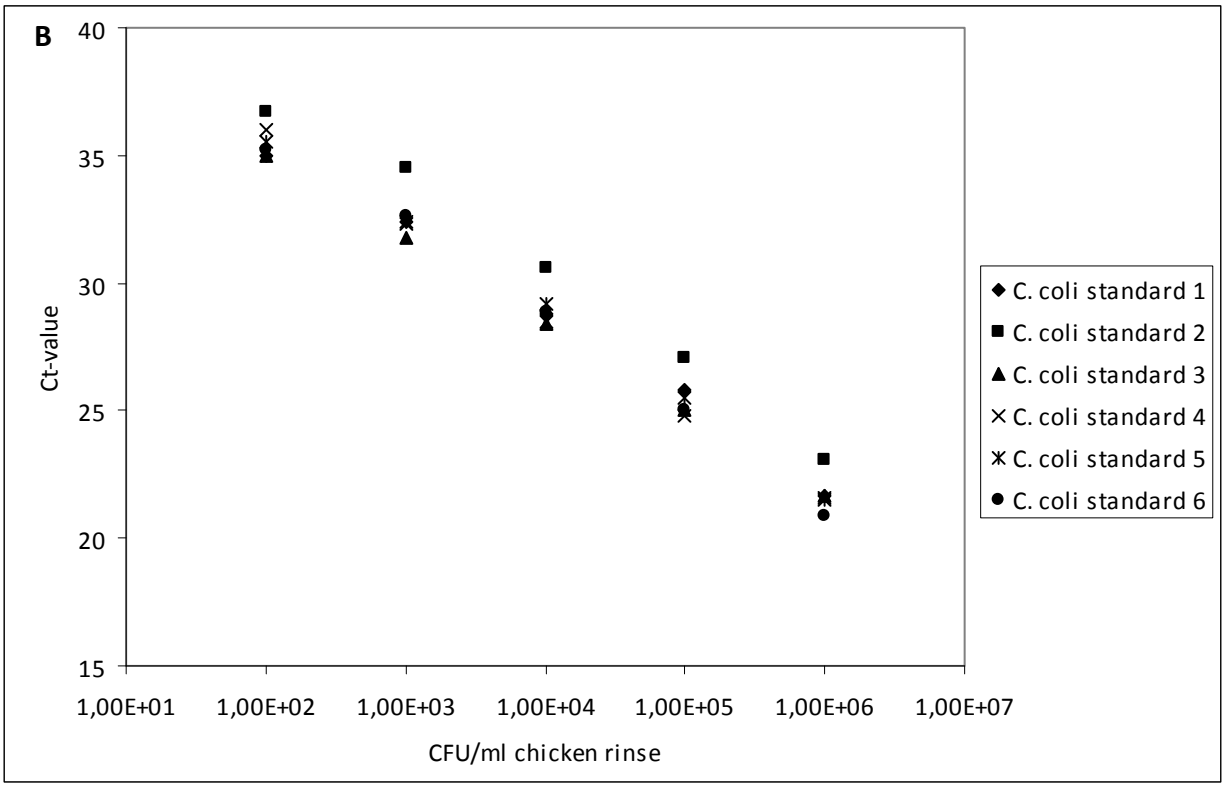

B
40
35
30
25
20
15
Ct-value
1,00E+01
1,00E+02
1,00E+03
1,00E+04
1,00E+05
1,00E+06
1,00E+07
CFU/ml chicken rinse
C. coli standard 1
C. coli standard 2
C. coli standard 3
C. coli standard 4
C. coli standard 5
C. coli standard 6

Figure 2. Standard curves produced from ten-fold serial dilutions ranging from 1×10^{-4} to 1×10^{1} ng DNA/PCR of *C. jejuni* CCUG 12795 and 14539, *C. coli* CCUG 10951 and 12791 and *C. lari* CCUG 20707 and 23947, showing the variation that can be ascribed to the species on DNA level. Each data point represents two Q-PCR replicates.

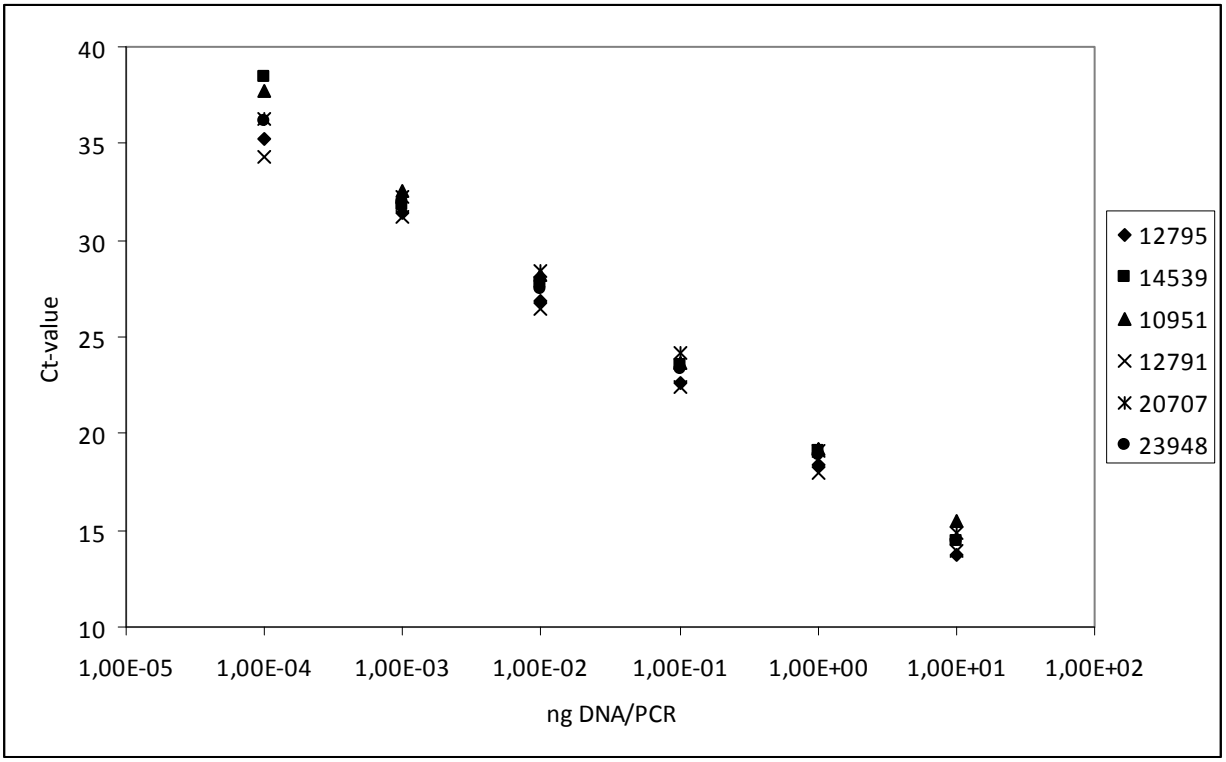


Figure 3. Standard curves produced from ten-fold serial dilutions ranging from 1×10^1 to 1×10^6 CFU/ml of *C. jejuni* CCUG 11284 (n = 4), *C. coli* CCUG 11283 (n = 2) and *C. lari* CCUG 23947 (n = 2), showing the variation that can be ascribed to the species on cell level. Not to camouflage the variation between species ΔCt-values were applied to standardize the amount of input cells, by subtracting the Ct-value for each cell level from the Ct-value obtained for 1×10^6 CFU/ml. Each data point represents two biological and two Q-PCR replicates.

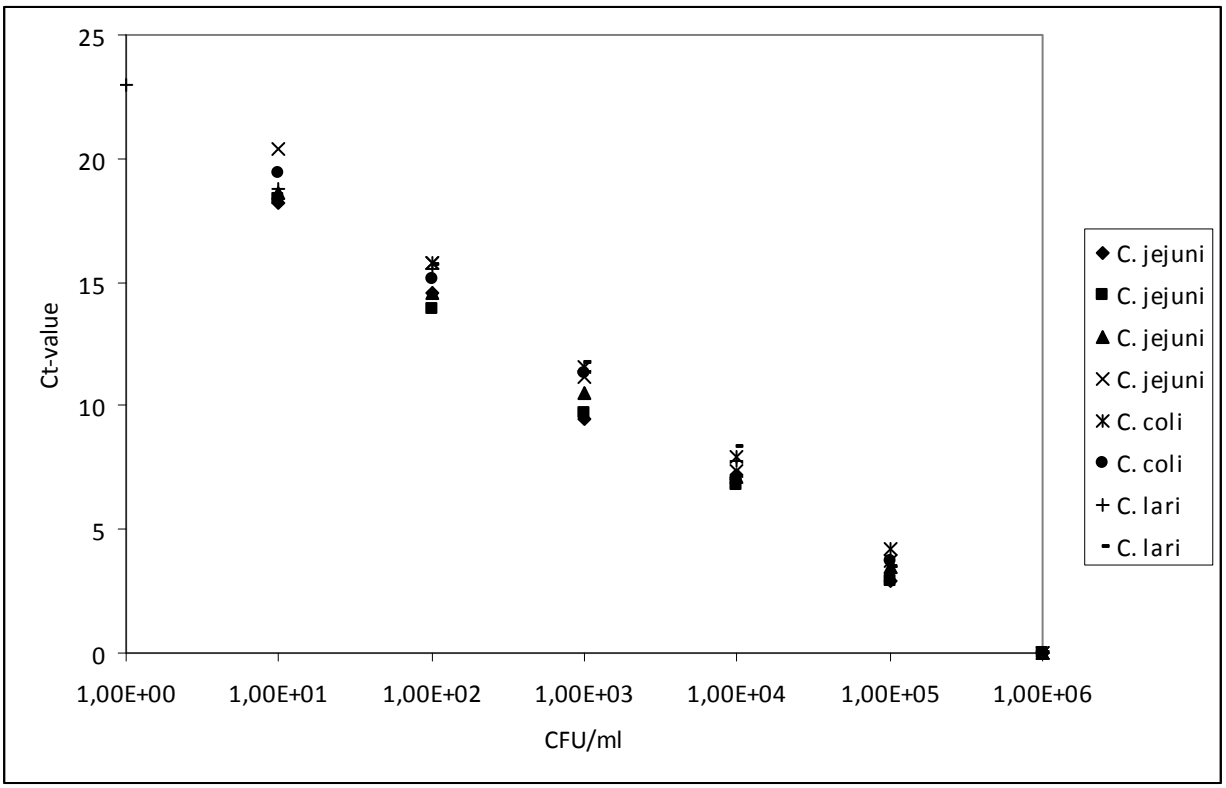


Figure 4. Correlation of *Campylobacter* quantitative results obtained by Q-PCR with **(A)** and without **(B)** PMA-treatment and culture on naturally contaminated chicken carcass rinse samples.